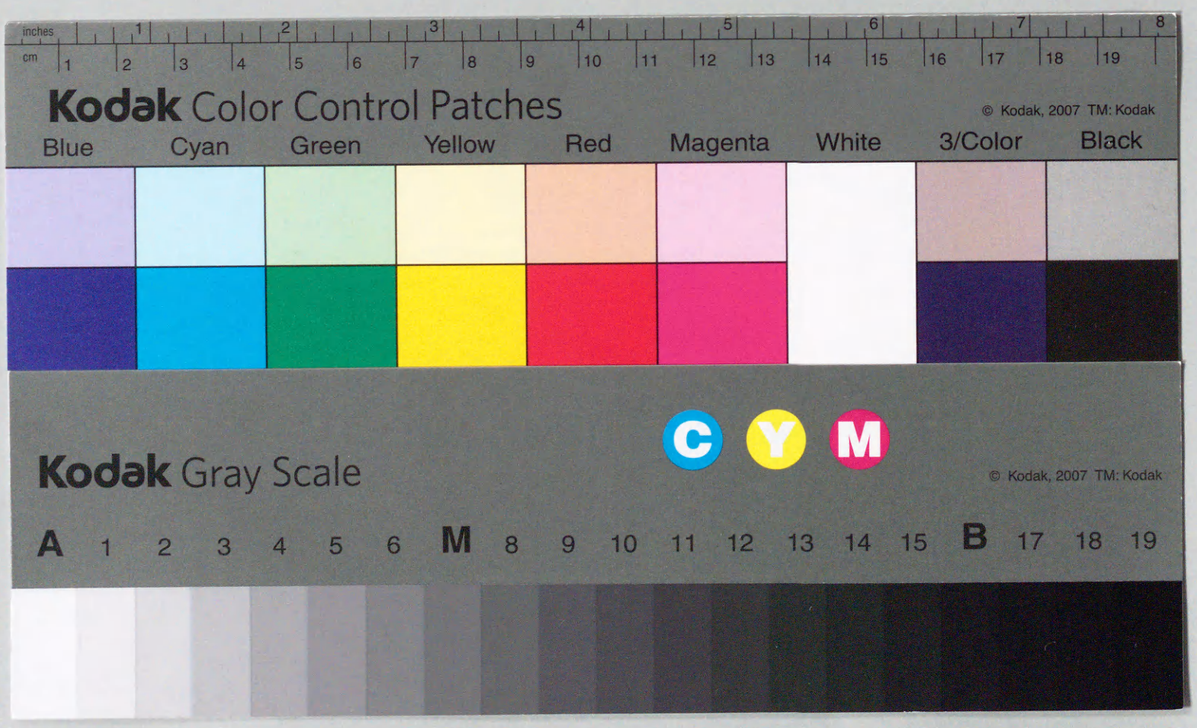
ROLES OF STIMULATORY G PROTEIN IN THE ENHANCEMENT OF RESPONSIVENESS TO ADRENALINE AND GLUCAGON

TATSUROU YAGAMI

1994



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List of Abbreviations

Bmax	number of maximal binding sites
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
CYP	cytochrome P-450
DTT	dithiothreitol
EGTA	ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
G protein	guanine nucleotide-binding regulatory protein
G_i	G protein that mediates inhibition of adenylate cyclase
$G_{i\alpha}$	α subunit of Gi
G_q	G protein that mediates stimulation of phospholipase C
G_s	G protein that mediates stimulation of adenylate cyclase
$G_{s\alpha}$	α subunit of G_s
$G_{s\alpha\text{-}S}$	small form of $G_{s\alpha}$
$G_{s\alpha\text{-}L}$	large form of $G_{s\alpha}$
Gt	G protein that mediates stimulation of cGMP phosphodiesterase
$G_{t\alpha}$	α subunit of Gt
GTPγS	guanosine-5'-*O*-(3-thio)triphosphate
Gpp(NH)p	guanosine-5'-[β, γimido]triphosphate
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
IBMX	3-isobutyl-1-methyl-xanthine
IC50	concentration giving 50% inhibition
ICYP	iodocyanopindolol
K_d	dissociation constant
kDa	kilo Dalton
K_i	inhibition constant
K_H	dissociation constant of R_H
K_L	dissociation constant of R_L
Mops	3-(*N*-morpholino)propanesulfonic acid
R_H	high affinity binding sites of a receptor for an agonist
R_L	low affinity binding sites of a receptor for an agonist
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDSa	sodium *N*-lauroyl salcosinate
TPCK	*N*-tosyl-L-phenylalanyl chloromethyl ketone

Chapter I

Introduction

Information transfer at the cell surface is very important in order for the cell to adjust its metabolism according to environmental changes. The role of the cell-surface systems in converting extracellular signals into changes in cellular milieu through intracellular second messengers was first described by Sutherland and his coworkers in 1956 (1). In 1971, Rodbell and his coworkers showed that, in conjunction with hormones, GTP regulates the signal transduction pathway that uses the enzyme adenylate cyclase to convert ATP into the intracellular second messenger cAMP (2). Nonhydrolyzable analogs of GTP such as Gpp(NH)p were shown to stimulate adenylate cyclase persistently (3), and activation of hormone receptors increased this rate of stimulation (4). From these studies, Rodbell inferred two central concepts. First, the signal transduction system comprises three-components; a receptor which specifically binds the hormone, a mediator that dispatches the second messenger, and an effector enzyme. Second, the GTP-binding site is a GTPase, since GTP is hydrolyzed between the β and γ phosphates and unhydrolyzable GTP analogs stimulate adenylate cyclase persistently even in the absence of hormones.

The discovery and subsequent characterization of guanine nucleotide-binding regulatory proteins (G proteins) (5-7) confirmed the above hypothesis. The G protein is a heterotrimeric protein composed in order of decreasing mass of α (G_α), β, and γ subunits (8, 9). The α subunit contains a single, high-affinity binding site for guanine nucleotides and possesses the GTPase activity that is crucial for the action of G proteins (10). Usually, the G_α determines the specificity of the interaction between the receptor and its effector. Accordingly, several distinct types of G_α subunits exist. For example, the α subunit ($G_{s\alpha}$) of stimulatory G protein (G_s), which stimulates adenylate cyclase in response to various

stimulatory agonists (*e.g.* β-adrenergic agonists and glucagon) (11); the α subunit ($G_{i\alpha}$) of inhibitory G protein (G_i), which inhibits adenylate cyclase in response to inhibitory agonists such as α_2-adrenergic agonists (11); and the α subunit ($G_{q\alpha}$) of G_q, which stimulates phospholipase C (12, 13). G_α also contains the site(s) for NAD-dependent ADP-ribosylation catalyzed by bacterial toxins. $G_{i\alpha}$ can be ADP-ribosylated by pertussis toxin (also known as islet-activating protein) (14). Two forms of $G_{s\alpha}$, with apparent molecular masses of 42-45 kDa ($G_{s\alpha\text{-}S}$) and 47-52 kDa ($G_{s\alpha\text{-}L}$), have been identified in numerous tissues by their ability to be ADP-ribosylated by cholera toxin (15, 16).

The mechanisms of signal transduction by G proteins have been studied in the adenylate cyclase systems by Gilman *et al.* (7-9). This cycle is summarized in Fig. 1. In the basal state, G proteins exist in their trimeric form with GDP tightly bound to G_α (7-9). The interaction of the G protein with an appropriately liganded receptor stimulates the dissociation of GDP, presumably as a result of a conformational change that results in an "opening" of the guanine nucleotide-binding site (17, 18). The hormone-receptor-G protein complex is apparently a relatively stable intermediate in the absence of GTP, and there have been several reports of the copurification (at least for initial steps) of a nucleotide free hormone-receptor-G protein complex (19, 20). However, in the presence of the high cellular concentrations of GTP, the "open" guanine nucleotide site is rapidly filled (11). The binding of GTP to hormone-receptor-G protein complex decreases the affinity of receptor for hormone and G protein (11). Dissociation of hormone and GTP-liganded G protein from receptor allows recycling of the receptor and the activation of G proteins (21). Such activation greatly reduces the affinity of the GTP-liganded α subunit (G_α-GTP) for the βγ complex, resulting in dissociation of the subunit. The G_α-GTP and the βγ complex can stimulate or inhibit

appropriate effectors (22). The G_α-GTP is transformed to G_α-GDP by the intrinsic GTPase activity of G_α-GTP, resulting in the reassociation of G_α-GDP with $\beta\gamma$ and terminating the signal transduction (23).

The adenylate cyclase/cAMP system to adrenaline or glucagon is altered in many physiological conditions such as aging (24). Moreover, abnormality of this system leads to various diseases including diabetes(25), hypertension (26), cancer (27) or asthma (28). The abnormal response of adenylate cyclase activity to these hormones may be attributed to alterations in receptors, G proteins or catalytic subunit of adenylate cyclase, or the combination of all three. It is well established phenomenon that the above physiological and pathological conditions are associated with changes in the quality and/or the quantity of receptors (29-33). Less attention has been paid to alterations in G proteins as compared to those in receptors. However, alterations in the function and expression of G proteins can contribute to the pathophysiology of human diseases. The notable examples are cholera (34) and pertussis (35). Cholera and pertussis toxins stimulate adenylate cyclase persistently by the ADP-ribosylation of $G_{s\alpha}$ and $G_{i\alpha}$, respectively, and elevate intracellular cAMP level (11). The external level of cAMP causes hypohydremia in intestine (34) or whooping cough in bronchia (35). Thus, it is important to clear how G proteins are involved in the abnormality of receptor-mediated functions.

One of the most extensively studied G protein-coupled receptors is adrenergic receptors. There are both α and β-adrenergic receptors in rat livers (36). Relative contribution of these two types of adrenergic receptors to adrenaline-induced glycogen break down is dependent on aging (24), sex (37), function of the thyroid (38) or adrenal (39) gland and regenerating state of liver after partial hepatectomy (40). Hepatic adenylate cyclase activity responding to β-adrenergic agonists is

significantly higher in the female than in the male (37). However, the content and the type of β-adrenergic receptors are identical between both sexes (41). Does the post-receptor adenylate cyclase system in the female differ from that in the male? To test the possibility, whether there is a sex difference in the function and/or the amount of $G_{s\alpha}$ was investigated in rat livers (Chapter II).

The human $G_{s\alpha}$ gene is isolated from human genomic libraries with rat $G_{s\alpha}$ cDNA (42). The gene contains 13 exons and 12 introns and spans about 20 kb of genomic DNA. Four different $G_{s\alpha}$ cDNAs ($G_{s\alpha\text{-}1}$ to $G_{s\alpha\text{-}4}$) is isolated from human brain (43). $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}3}$ are identical except that $G_{s\alpha\text{-}3}$ lacks a single stretch of 45 nucleotides. $G_{s\alpha\text{-}2}$ and $G_{s\alpha\text{-}4}$ have 3 additional nucleotides (CAG) compared to $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}3}$ located 3' to the above 45 nucleotides. Comparison of the four types of human $G_{s\alpha}$ cDNA (43) with the sequence of the human $G_{s\alpha}$ gene (42) suggests that four types of $G_{s\alpha}$ mRNAs may be generated from a single $G_{s\alpha}$ gene by alternative splicing as shown in Fig 2. $G_{s\alpha\text{-}1}$ has a sequence identical to exon 2, 3 and 4, whereas $G_{s\alpha\text{-}3}$ lacks exon 3. $G_{s\alpha\text{-}2}$ and $G_{s\alpha\text{-}4}$ have 3 additional nucleotides (CAG) to $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}3}$, respectively, at the 5' end of exon 4. In the preset study, $G_{s\alpha\text{-}1}$ and $G_{s\alpha\text{-}2}$ are referred as $G_{s\alpha\text{-}L}$, and $G_{s\alpha\text{-}3}$ and $G_{s\alpha\text{-}4}$ are as $G_{s\alpha\text{-}S}$. Whether these structural differences result in functional differences of the two $G_{s\alpha}$ is not yet clear.

Alterations in catecholamine-sensitive adenylate cyclase activity are accompanied with changes in the relative content of the two $G_{s\alpha}$, indicated by the following reports. First, the β-adrenergic adenylate cyclase system in rat erythrocyte decreases during reticulocyte maturation. Concurrently, the ratio of the amount of $G_{s\alpha\text{-}L}$ to that of $G_{s\alpha\text{-}S}$ decreases responding to attenuation of β-adrenergic responsiveness (44). Second, in a purification of rabbit liver G_s, catecholamine-stimulated adenylate cyclase activity in S49 cyc^-, which is the mutant of

murine lymphoma cell line S49, membranes reconstituted by $G_{s\alpha\text{-}L}$-enriched fractions is higher than those by $G_{s\alpha\text{-}S}$-enriched fractions (45). Extracts from transformed lung fibroblast cell lines which contain only $G_{s\alpha\text{-}L}$ are able to restore cholera toxin, fluoride, guanine nucleotide and hormone-stimulated adenylate cyclase activity to S49 cyc^- cells (46). Thus, $G_{s\alpha\text{-}L}$ appears to be coupled with β-adrenergic receptors more efficiently than $G_{s\alpha\text{-}S}$. Further studies are required to prove the functional difference of the two $G_{s\alpha}$. However, β-adrenergic responsiveness in the female was not high enough to analyze interactions between β-adrenergic receptors and G_s in detail. After partial hepatectomy, catecholamine-sensitive hepatic adenylate cyclase activity is markedly increased in rat livers (40). Actually, β-adrenergic responsiveness in partially hepatectomized male rats was about 5-fold higher than that in female rats, and enough high to examine receptor-G protein interaction. Hence, to identify the type of $G_{s\alpha}$ specific for β-adrenergic receptors, coupling of β-adrenergic receptors with G_s was evaluated in liver plasma membranes from partially hepatectomized male rats (Chapter III). In regard to functional significance of the multiple forms of $G_{s\alpha}$, it is possible that different cAMP generating receptors may couple different forms of $G_{s\alpha}$, respectively. Besides β-adrenergic receptors, glucagon receptors also stimulate adenylate cyclase *via* G_s (11). To test the possibility, the coupling of β-adrenergic and glucagon receptors with G_s were compared in partially hepatectomized male rat livers (Chapter IV). Finally, the present results are discussed comprehensively (Chapter V).

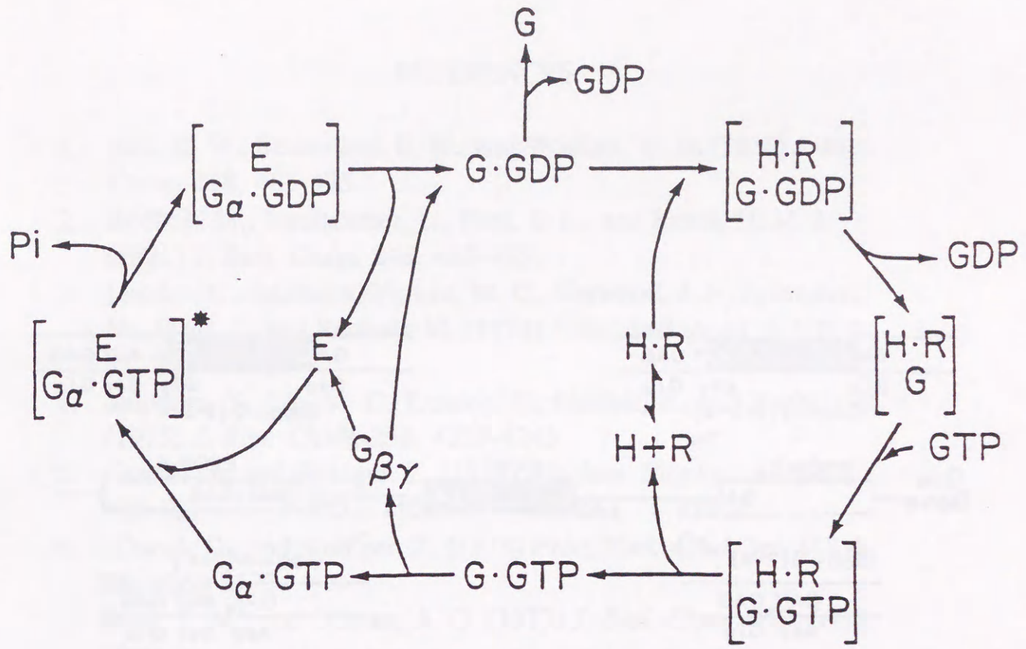

Fig. 1. **Interactions of hormone, receptor, G protein, guanine nucleotide, and effector (Ref. 11).** E, effector; G, G protein oligomer ($\alpha\beta\gamma$); G_α, α subunit of G protein; $G\beta\gamma$, complex of β and γ subunit of G protein; H, hormone; R, receptor; $[G_\alpha\cdot GTP\cdot E]^*$, activated form of effector. The low-affinity form of the receptor (H·R) interacts with the GDP-liganded heterotrimeric G protein (G·GDP) to form the high-affinity state of the receptor (H·R·G·GDP). H·R stimulates the release of GDP (H·R·G). GTP then binds to the nucleotide site on Gα (H·R·G·GTP), promoting the release of the G protein from H·R and the dissociation of the $G_{\beta\gamma}$ from G_α. The GTP-liganded and active G_α ($G_\alpha\cdot GTP$) then modulated the activity of the effector (E). The GTP is hydrolyzed by the intrinsic GTPase activity to form $G_\alpha\cdot GDP$, which has high affinity for $G_{\beta\gamma}$ and associates with it to form the heterotrimeric resting state of the G protein (G·GDP). The hormone receptor, upon dissociation of $G_\alpha\cdot GTP$, reverts to the low affinity state until it encounters another G·GDP.

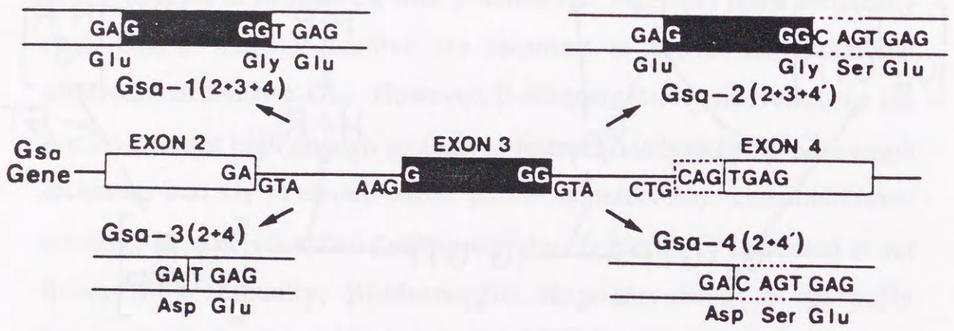

Fig. 2. **Model for origin of four different $G_{s\alpha}$ mRNAs by alternative splicing (Ref. 42).** The $G_{s\alpha}$ gene is shown in the center. Exon 2 and 4 are shown by open boxes, exon 3 is shown by solid box, and additional nuicleotides (CAG) are shown by dotted box. Nucleotide sequences of exon-intron boundaries are shown. Four $G_{s\alpha}$ mRNAs are indicated by $G_{s\alpha\text{-}1}$, -2, -3, and -4. $G_{s\alpha\text{-}1}$ has a sequence identical to exon 2, 3 and 4, whereas $G_{s\alpha\text{-}3}$ lackes a stretch of 45 nucleotides of $G_{s\alpha\text{-}1}$, which coincides with exon 3. $G_{s\alpha\text{-}2}$ has 3 additional nucleotides (CAG) to $G_{s\alpha\text{-}1}$ at the 3' end of the above 45 nucleotides. $G_{s\alpha\text{-}4}$ also has the 3 additional nucleotides to $G_{s\alpha\text{-}3}$ between exon 2 and 4.

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Chapter II

Alterations in Stimulatory G protein Associated with the Sex-Dependent Appearance of β-Adrenergic Receptor-Mediated Function in Rat Liver

INTRODUCTION

In rat liver, several distinct subtypes of the catecholamine receptor have been identified, based on their physiological actions and pharmacological specificities. These include the α_1-, α_2-, β_1- and β_2-adrenergic receptor subtypes (1). These different receptor subtypes are coupled to distinct effectors. Thus, α_1-adrenergic receptors activate phospholipase C through G_i (2) and G_q (3, 4), leading to the generation of second messengers such as diacyl glycerol and inositol triphosphate (5). On the other hand, α_2-adrenergic receptors inhibit adenylate cyclase *via* Gi (6). In contrast, both β_1- and β_2- adrenergic receptors stimulate adenylate cyclase *via* G_s, leading to the generation of cAMP (6). β-adrenergic receptors are predominantly β_2-type in normal rat livers (7). The type of β-adrnergic receptors is converted from β_2- to β_1-type under pathological conditions such as cancer (8, 9).

In rat liver, adrenaline can regulate glycogenolysis *via* both α_1- and β-adrenergic receptors (10). In adult male rats, adrenaline-induced glycogenolysis in the liver proceeds selectively *via* α_1-adrenergic receptors (11). However, the hepatic adrenergic mechanism has been shown to be converted from an α_1- to a β-type-mediating function following thyroidectomy (12), adrenalectomy (13), partial hepatectomy (14), cholestatis (15) and primary culturing of hepatocytes (16). This conversion is due primarily to the reciprocal changes in adrenergic receptors from the α_1- to β-type. Furthermore, the emergence of β-adrenergic response is also dependent on sex (17). The hepatic adenylate cyclase activity responding to β-adrenergic agonists is higher in adult female rats than that in male (17). On the other hand, there is no sexual dimorphism of α_1-adrenergic agonists-induced phospholipase C activity

(17). Nevertheless, there is no sex-dependent difference in the quality or quantity of not only α_1-adrenergic receptors but also β-adrenergic receptors (18). Therefore, the post-receptor adenylate cyclase system, *e.g.* G_s and/or the catalytic unit, might be one of the regulatory sites for development of the observed sexual dimorphism of β-adrenergic function. Indeed, the enhanced coupling of β-adrenergic receptors with G_s can contribute to the emergence of β-adrenergic function after adrenalectomy (19) and cholestatis (20). The present study aims to examine whether there is a sex difference in the amount of G_s and/or the intrinsic activity of adenylate cyclase.

EXPERIMENTAL PROCEDURES

Materials --- [*Adenylate* α-^{32}P]NAD (29.6 TBq/mmol) and [^{125}I]ICYP (8.14 TBq/mmol) were purchased from Du-Pont New England Nuclear. Aprotinine, adrenaline bitartrate, isoproterenol bitartrate, phenylephrine hydrochloride, prazosin hydrochloride, propranolol hydrochloride, yohimbine hydrochloride, forskolin, cholera toxin and IBMX were obtained from Sigma Chemical Company. Gpp(NH)p, GTPγS and NAD were from Boehringer Mannheim. Testosterone propionate and 17β-estradiol were from Nacalai Tesque. X-ray film (New-XR), film cassettes and intensifying screen were from Fuji Photo Film. Percoll was purchased from Pharmacia. Assay kits for cAMP were from Yamasa Shoyu. Pertussis toxin was from Kaken Seiyaku. All other chemicals were reagent grade and obtained from commercial sources.

Animals and Operations --- Male and female Slc:Wistar strain rats (9-10 weeks old), weighing 230-260 g and 140-170 g, respectively, were obtained from Japan SLC, Inc. Animals were housed in a

temperature- (21-25 °C) and light- (8:00 a.m. to 8:00 p.m.) controlled room and received food and water *ad libitum*. Castration was performed under sodium pentobarbital anesthesia (65 mg / kg rat). Castrated male and female rats were injected subcutaneously (500 µg / kg /day) testosterone propionate or 17β-estradiol for 10 days or with vehicle alone (100 µl of sesame oil) and sacrificed 1 day after the last injections. All surgical procedures were performed between 8:00 a.m. and noon.

Preparation of Isolated Rat Hepatocytes --- Liver parenchymal cells from fed rats were prepared according to the method of Tanaka *et al.* (21). The viability of the hepatocytes thus obtained routinely was 90-99 % according to monitoring by trypan blue exclusion. Hepatocytes were suspended at 3 x 10^6 cells/ml in Hepes-buffer medium consisting of 10 mM Hepes (pH 7.4), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$ and 5 mM $NaHCO_3$. This medium was supplemented by 40 mM glucose.

Preparation of Plasma Membranes from Rat Livers --- Liver plasma membranes were prepared according to the method of Lynch *et al.* (22) with a slight modification. Livers were perfused with saline and then removed quickly. After washing with saline, pooled livers (about 50 g) from 5 adult rats were minced and homogenized in 3 volumes of ice cold STEA solution consisting of 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1 mM EGTA, and 50 karikllein inhibitor units/ml aprotinine. The homogenate was filtered through three meshes and centrifuged at 700 x g for 10 min. The pellet was resuspended in 120 ml of STEA solution by gentle homogenization, and the resuspension was dispersed in 1080 ml of isosmotic Percoll solution (15.7 % Percoll in STEA solution). The mixture was centrifuged at 35,000 x g for 30 min. The second band from the surface was collected, washed by dilution with 2-3 volumes of HEA solution consisting of 25 mM Hepes-NaOH (pH 7.4),

1 mM EGTA, and 50 karikllein inhibitor units/ml aprotinine and centrifuged at 10,000 x g for 30 min. The pellet was suspended in HEA solution and stored in liquid nitrogen until used. The protein concentration of the membrane fractions were determined by the method of Schaffner and Weissman (23) using bovine serum albumin as a standard.

Assay of Adenylate Cyclase Activity --- Adenylate cyclase activity in isolated hepatocytes and liver plasma membranes was measured according to the method of Itoh *et al.* (24). The cell suspension (3 x 10^6 cells/ml) was first incubated for 15 min at 37 °C in the Hepes-buffer medium consisting of 10 mM Hepes (pH 7.4), 134 mM NaCl, 4.7mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$ and 5 mM $NaHCO_3$) in the presence of 400 µM IBMX under an atmosphere of 95 % O_2 - 5 % CO_2. After preincubation, 500 µl of the resulting cell suspension was further incubated with 10 µl of a test drug dissolved in Hepes-buffer medium at 37 °C for 2 min. After the termination of the reaction by adding 50 µl of 1 N HCl, the cell suspension was centrifuged at 1,700 x g for 10 min. The resulting supernatant was used for the measurement of cAMP. When plasma membrane was used for the assay of adenylate cyclase activity, the reaction mixture consisting of 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 400 µM IBMX, 500 µM ATP, 5 mM phosphocreatine, and 50 units/ml creatine phosphate kinase. The assay was started by adding 20 µl of plasma membrane (20 µg of protein) to 180 µl of the reaction mixture. After the incubation at 30 °C for 5 min with drugs at various concentrations in the presence of 100 nM GTPγS, the reaction was terminated by adding 20 µl of 1 N HCl. The supernatant obtained by centrifugation at 1,700 x g for 10 min was used for the determination of adenylate cyclase activity. cAMP generated from

ATP in hepatocytes or liver plasma membranes was measured with cAMP assay kit (25).

ADP-Ribosylation of Liver Plasma Membranes --- ADP-ribosylation by cholera toxin was carried out by a modification of the method of Nakamura *et al.* (16). Plasma membranes (20 µg of protein) were incubated with cholera toxin (50 µg/ml) for 45 min at 30 °C in 100 µl of the reaction mixture consisting of 100 mM potassium phosphate (pH 8.0), 12.5 mM thymidine, 100 µM GTP, 1 mM ATP, 1 mM $MgCl_2$, 500 µM EGTA, and 20 µM [^{32}P]NAD (1.85 MBq/ml). Cholera toxin was activated with 20 mM dithiothreitol at 30 °C for 20 min before use. After incubation, membranes were collected by centrifugation at 7,000 x g for 10 min and pellets were suspended in 20 µl of sample buffer consisting of 125 mM Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, and 20 % glycerol, boiled for 3 min and subjected to SDS-PAGE with 11% gels. After electrophoresis, the gels were soaked in 15% trichloroacetic acid, dried, and exposed to X-ray film using an intensifying screen for 2-9 days at -80 °C. For quantitation, dried gels were exposed to a Fujix imaging plate and analyzed by a Fujix Bioimaging analyzer BAS 2000 instrument.

ADP-ribosylation by pertussis toxin was carried out by a modification of the method of Itoh *et al.* (24). Plasma membranes (20 µg of protein) were incubated with pertussis toxin (2 µg/ml) for 90 min at 30 °C in 25 µl of the reaction mixture consisting of 20 mM Tris-HCl (pH 7.4), 10 mM thymidine, 10 mM NADP, 1 mM EGTA, and 2 µM [^{32}P]NAD (1.48 MBq/ml). Pertussis toxin was activated with 1 mM dithiothreitol and 0.5 mM ATP at 30 °C for 20 min before use. After incubation, membranes were treated by the same method as cholera toxin-catalyzed ADP-ribosylation.

Binding Study of [^{125}I]ICYP in Liver Plasma Membranes --- The binding assay was performed according to the method of Nakamura *et al.* (26) with a minor modification. The assay mixture consists of 50 mM Hepes (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM sodium ascorbate, 1 mM EGTA, 1 mM pyrocatechol, 50 kallikrein inhibitor units/ml aprotinine, and liver plasma membranes (100 µg of protein). 250 µl of assay mixture was incubated at 25 °C for 40 min in the presence or absence of 10 µM GTPγS with 100 pM [^{125}I]ICYP and the indicated concentrations of isoproterenol. The reaction was terminated by rapidly diluting with 5 ml of ice-cold TMN buffer consisting of 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 100 mM NaCl. The dilute samples were immediately filtered under vaccume through Whatman GF/C glass fiber filters (2.4 cm), which had been presoaked with TMN buffer containing 1 mM sodium ascorbate and 1 mM pyrocatechol. The filters were promptly washed three times with 2.5 ml of ice-cold TMN buffer. Radioactivity on the filter was measured in an Aloka ARC 600. Nonspecific binding was determined in the presence of 10 µM d,l-propranolol, which was routinely less than 20 %.

RESULTS

Effects of Adrenergic Agonists on cAMP Accumulation in Hepatocytes --- Using hepatocytes isolated from male and female rats, β-adrenergic receptor-mediated cAMP accumulation was examined (Fig. 1). There was no significant difference in the basal level of accumulated cAMP in either males or females. Adrenaline increased cAMP accumulation in a concentration-dependent manner. However, the increment in cAMP accumulation responding to 10 µM adrenaline was about two times higher in the female than that in the male. In females, a

β-adrenergic agonist, isoproterenol, was more effective for stimulating adenylate cyclase than an α_1-adrenergic agonist, phenylepherine. On the other hand, these adrenergic agonists exhibited slight or no stimulatory effect on cAMP accumulation in the male. Adrenergic antagonists inhibited adrenaline-induced cAMP accumulation (Table I). The inhibitory effect of propranolol, a β-adrenergic antagonist, was stronger than that of prazosin, an α_1-antagonist, or yohimbine, an α_2-antagonist. These results has confirmed that the catecholamine-sensitive cAMP generation system in rat hepatocytes is functionally coupled to β-adrenergic receptors (6), and that the β-adrenergic response is higher in females than that in males (17).

cAMP Generation in Liver Plasma Membranes --- To ascertain whether there is a sexual dimorphism in the post-receptor adenylate cyclase system, activities of adenylate cyclase in liver plasma membranes were compared between male and female rats (Fig. 2). No significant sexual difference in the basal activity of the enzyme was observed. GTPγS, a nonhydrolyzable GTP analog, is known to stimulate adenylate cyclase indirectly *via* G_s (6). Upon addition of this analog, cAMP generation was increased in a concentration-dependent manner. The maximal response of adenylate cyclase activity to GTPγS in females was about two times higher than that in males. On the other hand, forskolin, known to stimulate adenylate cyclase directly (27), also increased cAMP generation in a concentration-dependent manner. The maximal response of the enzyme activity to forskolin in the female was, however, similar to that in the male, indicating no sex difference in the intrinsic activity of adenylate cyclase. These results suggest that alterations in G proteins might be involved in the sexual dimorphism of the β-adrenergic response.

ADP-Ribosylation of G proteins by Bacterial Toxins --- To elucidate whether there is a sex-associated difference in the amount of $G_{s\alpha}$, liver plasma membranes were treated with cholera toxin to induce ADP-ribosylation and subjected to SDS-PAGE. The quantitative reliability of this method is similar to that of immunoblotting using antisera specific for $G_{s\alpha}$ (28). Cholera toxin catalyzed ADP-ribosylation of two protein bands (42 kDa and 47 kDa) specifically in plasma membrane (Fig. 3A); these were $G_{s\alpha\text{-}S}$ and $G_{s\alpha\text{-}L}$, respectively (6). The amounts of $G_{s\alpha\text{-}S}$ and $G_{s\alpha\text{-}L}$ were 1.5-times and 2.0-times larger, respectively, in the female than those in the male (Fig. 3B).

Next, to ascertain whether there is sexual dimorphism of the function of G_i, liver plasma membranes were treated with pertussis toxin to induce ADP-ribosylation and subjected to SDS-PAGE. Pertussis toxin catalyzed ADP-ribosylation of a protein band (41 kDa) specifically in plasma membranes (Fig. 3A), corresponding to $G_{i\alpha}$ (25). The amount of $G_{i\alpha}$ in the female was similar to that in the male (Fig. 3B).

Competitive Binding Study of β-Adrenergic Agonist in Liver Plasma Membranes --- The coupling of receptor with G proteins may regulate the affinity of receptor binding for agonists, but not for antagonists (29). A complex of β-adrenergic receptor and GDP-bound G_s exhibits higher affinities for the agonists than β-adrenergic receptors alone (29). Upon the exchange of GTP for the bound GDP, G_s is released from β-adrenergic receptors and the resultant G_s-free β-adrenergic receptors become low-affinity receptors for the agonists (30). To examine whether there are differences in the coupling of β-adrenergic receptors with G_s between male and female, competitive binfding study of an agonist with an antagonit was performed in the presence or absence of GTPγS. As shown in Fig. 5, inhibiting curves of [^{125}I]ICYP binding by isoproterenol in liver plasma membranes from male gave IC_{50} values of

580 ± 30 nM or 71 ± 5 nM in the presence or absence of GTPγS, respectively . On the other hand, in the female, the IC_{50} values of isoproterenol in the presence or absence of GTPγS were 998 ± 50 nM and 32 ± 3 nM, respectively. With Scatchard analysis (31) of the data in Fig. 5, the proportion of β-adrenergic receptors in the high-affinity (R_H) and the low-affinity (R_L) state was evaluated (Table II). The proportion of high-affinity sites for isoproterenol in female rats was higher than that in male rats. On the other hand, the high-affinity values (K_H) of β-adrenergic receptors for their agonist were identical in both sexes. Since the high-affinity state of β-adrenergic receptors is produced by the receptor-G_s complex (29, 30), these results indicated that the proportion of β-adrenergic receptors coupled with G_s increased in parallel with the emergence of β-adrenergic responsiveness.

Binding Studies of β-Adrenergic Antagonists in Liver Plasma Membranes --- To further ascertain whether there are no sex-related differences in β-adrenergic receptors, the capacities for [^{125}I]ICYP binding to liver plasma membranes was examined. The potency of adrenergic antagonists as inhibitors of [^{125}I]ICYP binding in competitive experiments was propranolol > phentolamine, indicating β-characteristic of the binding site. Scatchard analyses are summarized in Table II. The number of β-adrenergic receptors in the female was about two times higher than that in the male. On the other hand, there was no significant difference in the K_d values. These results indicated that β-adrenergic receptors were up-regulated in females.

Effects of Sex Hormones on Isoproterenol-Induced cAMP Accumulation in Hepatocytes --- To test the possibility that sex hormones modulates β-adrenergic function, effects of sex hormones on isoproterenol-induced cAMP accumulation in hepatocytes were examined (Fig. 6). Neither castration nor administration of sex hormones changed

in the basal level of cAMP in either sex. In castrated male and female rats, isoproterenol-induced cAMP accumulation was not enhanced significantly as compared with that in normal rats. In testosterone propionate-administered male and female following castration, β-adrenergic responses were also similar to those in control rats. However, administration of 17β-estradiol increased more than 3-fold in the maximal response of hepatic adenylate cyclase to isoproterenol in both sexes. These results indicated that estrogen, but not androgen, has a potentiating effect on β-adrenergic responses.

DISCUSSION

Present study indicates that there are sex-dependent differences in the amount of $G_{s\alpha}$ and the coupling of β-adrenergic receptors with G_s. It was confirmed that hepatic adenylate cyclase activity responding to β-adrenergic agonists in the female is higher than that in the male (Fig. 1 and Table I). The sex difference in β-adrenergic adenylate cyclase system did not exist in the intrinsic activity of adenylate cyclase (Fig. 2B). However, the amount of $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$ was increased (Figs. 2A and 3) and the coupling of β-adrenergic receptors with G_s was enhanced (Fig. 4 and Table II) in the female. Furthermore, the ratio of $G_{s\alpha\text{-}L}$ to $G_{s\alpha\text{-}S}$ was greater in the female than in the male (Fig. 3). Thus, alterations in G_s appear to be closely correlated with the sex-dependent appearance of β-adrenergic responses.

Since catecholamines are capable of inhibiting adenylate cyclase *via* the α_2-adrenergic receptor-G_i pathway, the attenuation of G_i-mediated inhibition of the enzyme may be involved in the development of β-adrenergic function. Actually, in the primary culture of rat hepatocytes, the decrement in the amount of $G_{i\alpha}$, detected by pertussis toxin-catalyzed

ADP-ribosylation, contributes to the emergence of β-adrenergic sensitivity (24). This possibility, however, was not supported for the following reasons. Because hepatic adenylate cyclase activity responding to a selective β-adrenergic agonist was also higher in the female (Fig. 1). Furthermore, there was significant sexual dimorphism of adrenaline-induced cAMP accumulation in hepatocytes even in the presence of a selective α_2-adrenergic antagonist (Table I). Finally, the amount of $G_{i\alpha}$ was essentially the same regardless of the sex of the animal.

Studer *et al.* reported that the number of β-adrenergic receptors detected by [^{3}H]CGP-12177 was identical in crude membrane preparations from both sexes (17). [^{3}H]CGP-12177 is a hydrophilic β-adrenergic antagonist and can be used to measure for cell surface receptors (18). Hermsdorf *et al.* reported that the number of β-adrenergic receptors detected by [^{3}H]CGP-12177 was three times higher in hepatocytes, but not membranes, from female rats than those from male (33). For this discrepancy, they explained that the number of β-adrenergic cell surface receptors accessible for [^{3}H]CGP-12177 is significantly higher in the female, whereas there is no difference in the total amount of β-adrenergic receptors, including sequestered receptors, in either sex.

The possibility of the formation of inside-out vesicles cannot yet be ruled out during the membrane preparation (18, 33). This would lower the accessibility of the hydrophilic ligands to the receptors in crude membranes and make determination of the total amount uncertain. On the other hand, the hydrophobic probes are dissolved in the plasma membranes, distributed internally, and can also interact with the receptors inside vesicles (34). Therefore, hydrophobic ligands are more suitable for detecting the total amount of receptors than hydrophilic ligands. Thus, a hydrophobic β-adrenergic antagonist, [^{125}I]ICYP, was

used in this study to evaluate the total number of β-adrenergic receptors. The total amount of β-adrenergic receptors was significantly larger in the female than in the male (Table I). This result together with those of previous reports (18, 33) indicated that there was a sex difference not only in the number of accessible β-adrenergic cell-surface receptors but also in the total amount of β-adrenergic receptors. The order of potencies of agonists for binding to β-adrenergic receptors in membranes (isoproterenol > adrenaline > salbutamol > noradrenaline) indicated that the subclass of β-adrenergic receptors was the β_2-type in rat livers for both sexes. From these results, it seems reasonable to conclude that the enhanced coupling of β-adrenergic receptors with G_s is due to up-regulation of both receptors and G proteins rather than qualitative changes in the β-adrenergic receptors themselves.

Male and female rat livers have receptors for androgen (35) and estrogen (36), displaying a number of sexually dimorphic characteristics (37, 38). In weanling (39) and young female rats (40), injection of androgen showed a significant decrease in isoproterenol-induced adenylate cyclase activity. On the other hand, in castrated adult male and female rats, androgen had little effect on isoproterenol-induced cAMP accumulation in hepatocytes (Fig. 6). In contrast, in weanling (39) or castrated adult male rat livers (41), treatment with estrogen elevates the β-adrenergic response. In castrated adult male and female rats, administration of estrogen markedly increased β-adrenergic response (Fig. 6). Thus, estrogen appears to play an important role in the acquisition of β-adrenergic function.

The present results demonstrate that the sex-dependent appearance of β-adrenergic function can be ascribed to increases in the number of β-adrenergic receptors, the amount of $G_{s\alpha}$ and the interaction between the

receptors and G_s. Moreover, it seems likely that estrogen, but not androgen, is involved in the emergence of β-adrenergic function.

Table I

Effects of adrenergic antagonists on adrenaline-induced cAMP accumulation in hepatocytes

	Male	Female
No addition	2.02 ± 0.04	4.14 ± 0.16
100 nM Prazosin	1.86 ± 0.03	3.64 ± 0.12
100 nM Propranolol	0.73 ± 0.02	2.40 ± 0.10
100 nM Yohimbine	2.00 ± 0.03	4.89 ± 0.21
10 μM Yohimbine	1.27 ± 0.02	3.02 ± 0.11

Hepatocytes prepared from male or female rats were incubated with various adrenergic antagonists in the presence of 10 μM adrenaline. Values are the mean ± S.E. from 4 separate experiments.

Table II

Kinetic parameters for specific [^{125}I]ICYP binding to liver plasma membranes

	B max (fmol / mg protein)	K_d (pM)
Male	72.0 ± 5.4	129 ± 25
Female	163.2 ± 22.4*	151 ± 12

Membranes were prepared from male or female rats. Bmax and K_d values were determined from Scatchard analysis of [^{125}I]ICYP binding study. Values are the mean ± S.E. of 4 separate experiments. *P<0.05, for values of females *versus* males from t test.

Table III

Kinetic parameters for specific isoproterenol binding to liver plasma membranes

	Male	Female
K_L (nM)	330 ± 18	602 ± 31
K_H (nM)	5.2 ± 1.1	6.7 ± 1.9
K_L/K_H	68 ± 5	90 ± 6
R_L (%)	55 ± 5	45 ± 5
R_H (%)	45 ± 6	55 ± 6

Membranes were prepared from male or female rats. Competitive experiments were carried out as outlined in Fig. 4. The parameters were calculated by Scatchard analysis of the data in Fig. 4.Values are the mean ± S.E. of 4 separate experiments.

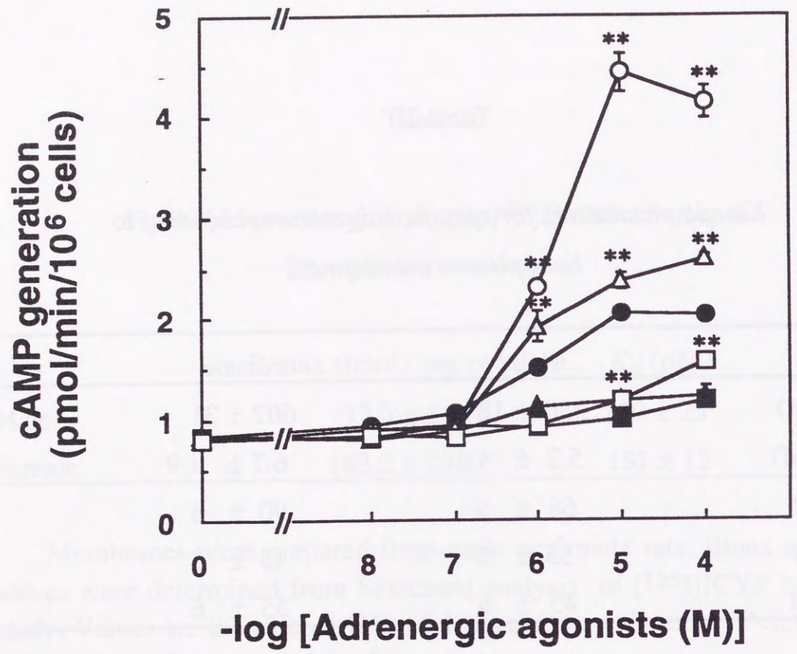

Fig. 1. **Effects of adrenergic agonists-induced cyclic AMP accumulation in hepatocytes.** Hepatocytes isolated from male (●, ▲, ■) or female (○, △, □) rats were incubated with indicated concentrations of adrenaline (●, ○), isoproterenol (▲, △) or phenylephrine (■, □). Values are the mean ± S.E. of 4 separate experiments. **P<0.01, for values of females *versus* males from t test.

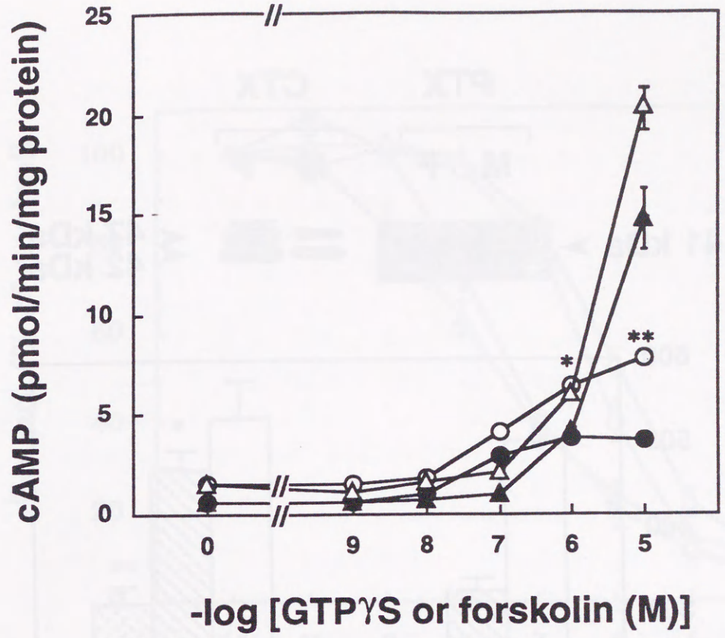

Fig. 2. **cAMP generation in liver plasma membranes.** Liver plasma membranes prepared from male (●, ▲) or female (○, △) rats were incubated in the presence of 400 μM IBMX with indicated concentrations of GTPγS (●, ○) or forskolin (▲, △) for 5 min at 30 °C. Values are the mean ± S.E. of 4 separate experiments. *P< 0.05 or **P<0.01, for values of females *versus* males from t test.

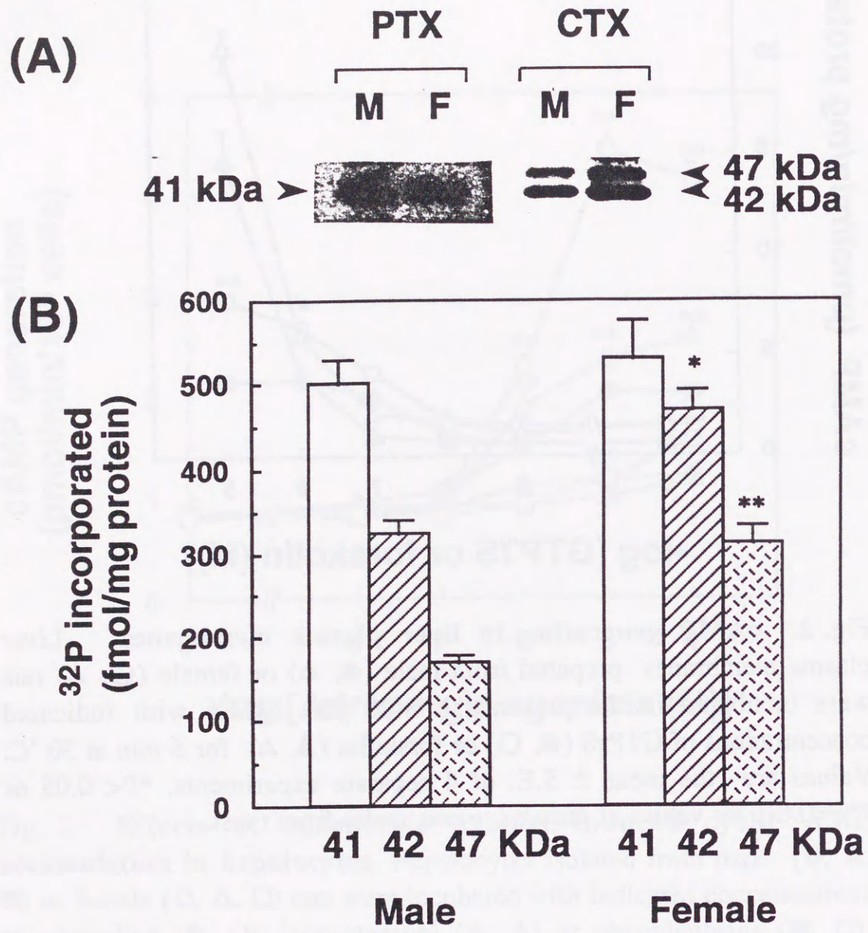

Fig. 3. **ADP-ribosylation of G proteins by bacterial toxins.** Autoradiogram of [^{32}P]ADP-ribosylated proteins of the $G_{i\alpha}$ and $G_{s\alpha}$ sites (A). Quantification of [^{32}P]ADP-ribosylated proteins of the Gi_{α} and $G_{s\alpha}$ site (B). Membranes were prepared from male (M) or female (F) rats. 41 kDa protein is a substrate for pertussis toxin (PTX). 42 and 47 kDa proteins are substrates for cholera toxin (CTX). Values are the mean ± S.E. of 4 separate experiments. *P<0.05 or **P<0.01, for values of females *versus* males from t test.

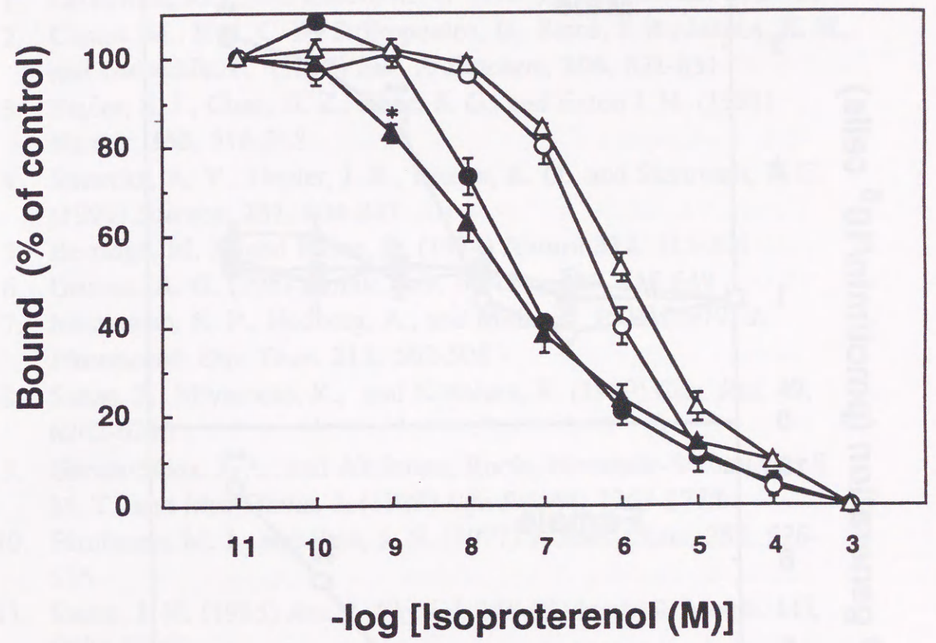

Fig. 4. **Inhibition of [^{125}I]ICYP binding by isoproterenol in liver plasma membranes.** Membranes from male (○, ●) or female (△, ▲) rats were incubated with 100 pM [^{125}I]ICYP and indicated concentrations of isoproterenol at 25 °C for 40 min, either in the absence (●, ▲) or presence (○, △) of 10 μM GTPγS. Values are the mean ± S.E. of 4 experiments. *P<0.05, for values of females *versus* males from t test.

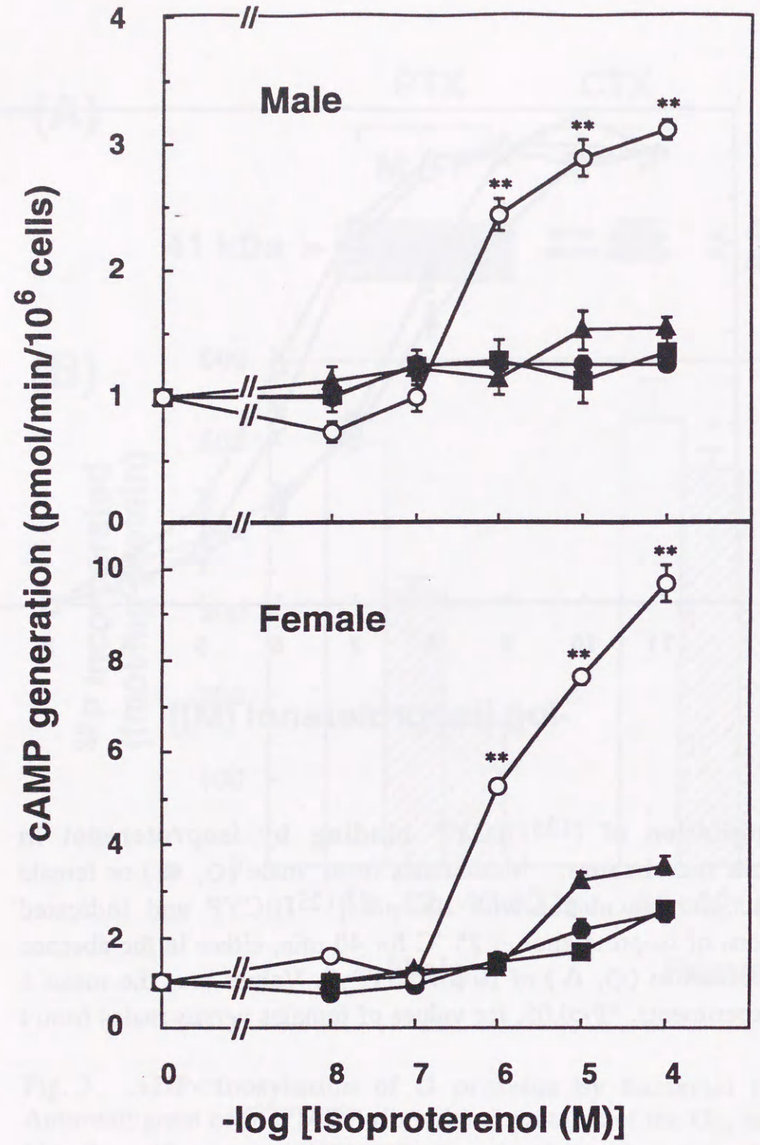

Fig. 5. **Effects of sex hormones on isoproterenol-induced cAMP accumulation in hepatocytes.** Hepatocytes were isolated from male (top panel) or female (bottom panel) rats. Rats were normal (●), castrated (▲), treated with testosterone propionate (■) or 17β-estradiol (○) following castration. Hepatocytes were incubated with the indicated concentrations of isoproterenol. Values are the mean ± S.E. of 4 separate experiments. **P<0.01, *versus* to normal rats from t test.

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Chapter III

Alterations in Stimulatory G protein Associated with the Partial Hepatectomy-Dependent Appearance of β-Adrenergic Receptor-Mediated Function in Rat Liver

INTRODUCTION

Based on the results from compositional analyses of the two $G_{s\alpha}$ related to β-adrenergic response, it was suggested that $G_{s\alpha\text{-}L}$ is more efficiently coupled to β-adrenergic receptors than $G_{s\alpha\text{-}S}$ (1-3). Reconstitution of receptor function in phospholipid vesicles from purified receptors and G proteins is the most common approach to determine which G protein is coupled with a given receptor. However, such approach was not undertaken to study functional significance of the two forms of G_s. $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$ expressed in *Escherichia coli* can reconstitute isoproterenol-, guanine nucleotide- and fluoride-stimulated adenylate cyclase activity in S49 cyc^- cell membranes, but neither forms of $G_{s\alpha}$ retain its coupling preference for β-adrenergic receptors (4, 5). Because of the nonphysiological environment around receptors and G proteins, this approach appears to have generated conflicting results (6, 7). Rat liver plasma membranes used in the present study more closely represent physiological conditions than phospholipid vesicles. Adenylate cyclase activity in response to catecholamines was higher in liver plasma membranes from female rats than those from male (Chapter II). However, this response was not high enough to analyze interactions between β-adrenergic receptors and G_s.

Partial hepatectomy in male rats develops feminization of several sexually dimorphic liver functions (8-10). In the female, the appearance of β-adrenergic responses is accompanied with increases in the number of β-adrenergic receptors, the amount of $G_{s\alpha}$ and the interaction between the receptors and Gs, without changes in the intrinsic activity of adenylate cyclase (Chapter II). Therefore, it is possible that partial hepatectomy increases β-adrenergic responses in male together with the concomitant

alterations in β-adrenergic receptors and G_s. Indeed, partially hepatectomized male rat livers acquire β-adrenergic function, and this function is markedly higher than that in females (11, 12). The number of β-adrenergic receptors increases during liver regeneration corresponding to enhancement of β-adrenergic function (12, 13). The subclass of β-adrenergic receptors is invariably the β_2-type throughout the process of liver regeneration (12). There is no significant alterations in the intrinsic activity of adenylate cyclase following partial hepatectomy (12). These results strengthen the possibility that the increment of β-adrenergic function after partial hepatectomy is also associated with the up-regulation of $G_{s\alpha}$ and the enhanced coupling of β-adrenergic receptors with G_s. Providing it is just like, the process of liver regeneration after partial hepatectomy can offer a suitable model to investigate the precise coupling pathways between β-adrenergic receptors and Gs. Thus, the possibility was tested in the present study.

In this study, a method using tryptic digestion was applied for evaluation of receptor-G protein interaction. This method was developed, based on the previous observation that GTP induces an alteration in the susceptibility of G_α to tryptic digestion (14). Since the agonist-receptor complex catalyzes the exchange of GDP for GTP on G_α, it is possible that hormonal stimulation affects the sensitivity of G_α to trypsin *via* its receptors. To investigate this possibility, liver plasma membranes from partially hepatectomized male rats were treated with trypsin following the stimulation by β-adrenergic agonists. Furthermore, with this method, the type(s) of G_s specific for β-adrenergic receptors was defined with this method.

EXPERIMENTAL PROCEDURES

Materials --- [*Adenylate* α-^{32}P]NAD (29.6 TBq/mmol) and [^{125}I]ICYP (8.14 TBq/mmol) were purchased from Du Pont-New England Nuclear. Adrenaline bitartrate, isoproterenol bitartrate, propranolol hydrochloride, cholera toxin, IBMX, TPCK-treated trypsin, soybean trypsin inhibitor and tamoxifen were obtained from Sigma Chemical Co. Pertussis toxin was from Kaken Seiyaku. GTPγS, Gpp(NH)p and NAD were from Boehringer Mannheim. X-ray film (New-XR), film cassettes, and intensifying screens were from Fuji Photo Film. The Megalabel™ kit and Oligotex™^{dT}-30 were from Takara. The assay kit for cAMP were from Yamasa Shoyu Co. All other chemicals were reagent grade and obtained from commercial sources.

Animals and Operations --- Male Slc:Wistar strain rats (9-10 weeks old), weighing between 230-260 g, were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Rats were housed in a temperature- (21-25 °C) and light- (8:00 a.m. to 8:00 p.m.) controlled room and fed *ad libitum*. Partial hepatectomy (70%) was performed according to the method of Higgins and Anderson (15). Animals were laporotomized along the median line under sodium pentobarbital anesthesia (65 mg/kg, intraperitoneal) and the left and median lobuli of the liver, which constituted 60 to 70 % of the liver, were resected from their basis. No special medication was given after the operation. The day of the operation was designated as Day 0. Sham hepatectomy consisted of laparotomy and gentle manipulation of the liver. In order to avoid variation due to circadian rhythm, all surgical procedures were performed between 8:00 a.m. and noon.

Measurement of Adenylate Cyclase Activity --- Rat hepatocytes were isolated according to the method of Tanaka *et al.* (16)

and as described in Chapter II. Liver plasma membranes were prepared according to the method of Lynch *et al.* (17) and as described in Chapter II. cAMP generated in rat hepatocytes and liver plasma membranes was measured according to the method of Itoh *et al.* (18) as described in Chapter II.

Binding study of [^{125}I] ICYP in Liver Plasma Membranes --- The binding study was performed according to the method of Nakamura *et al.* (19) as described in Chapter II.

ADP-Ribosylation of Liver Plasma Membranes by Bacterial Toxin --- ADP-ribosylations by cholera toxin and pertussis toxin were carried out by a modification of the method of Nakamura *et al.* (20) and the method of Itoh *et al.* (18), respectively, as described in Chapter II.

Northern Hybridization Analysis --- The 20-mer and 40-mer oligonucleotide probes which are complementary to the nucleotide sequences 237-256 of $G_{s\alpha\text{-}L}$ and 193-232 of $G_{s\alpha\text{-}S}$ (21, 22), respectively, were synthesized and endolabeled by polynucleotide kinase. Total RNA from normal and partially hepatectomized rats was isolated by the method of Chomczynski and Sacchi (23). Poly(A)$^+$ RNA was purified by oligo (dT) cellulose method (24). The concentration of RNA was spectrophotometrically estimated. Ten μg of poly(A)$^+$ RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose membranes. After UV crosslinking, the membranes were stained with 0.02 % methylene blue to check the quality of blotting. Prehybridization was performed at 57 °C for 4 h with hybridization buffer (5 x SSC, 10 x Denhart's solution, 0.1% SDSa, 10 mM EDTA, 300 μg/ml heat denatured salmon testis DNA, and 0 or 20 % formamide). Hybridization was carried out at 57 °C for 18 h with each of the oligonucleotide probes (1-2 x 10^6 cpm) in hybridization buffer. After

membranes were washed three times with a washing solution (5 x SSC, 0.1% SDSa, and 0 or 20 % formamide) at 57 °C for 20 min. They were exposed to X-ray film for autoradiography using an intensifying screen at -80 °C for 40 h. For quantitation, membranes were exposed to a Fujix imaging plate and analyzed by a Fujix Bioimaging Analyzer BAS 2000 instrument.

Tryptic Digestion of Liver Plasma Membranes --- Trypsin treatment of liver plasma membranes was performed essentially as described by Mazzoi *et al.* (14). The mixture (20 µl) containing 50 mM Hepes (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM EGTA, 50 kallikrein inhibitor units/ml aprotinine, and liver plasma membranes (20 µg of protein) prepared on the 2nd day after partial hepatectomy was preincubated at 30 °C for 20 min in the presence or absence of effectors. To the mixture was added 80 µl of a trypsin solution containing 125 mM Mops (pH 7.5), 225 mM NaCl, 5 mM $MgCl_2$, 1.1 mM dithiothreitol, and 12.5 µg/ml TPCK-trypsin and the mixture was further incubated at 30 °C. At the indicated times, the reaction was terminated by the addition of trypsin inhibitor with a trypsin-trypsin inhibitor ratio of 1:10 (w/w). The samples were washed with 10 mM Mops (pH 7.5), ADP-ribosylated by cholera toxin, and subjected to SDS-PAGE.

RESULTS

The Changes in Adrenergic Agonists-Induced Adenylate Cyclase Activity of Rat Liver after Partial Hepatectomy --- Using male rat hepatocytes, the changes in catecholamine-induced cAMP accumulation was examined after partial hepatectomy (Fig. 1). The basal

activity of adenylate cyclase was not altered throughout liver regeneration. On the other hand, after surgery, hepatic adenylate cyclase activity responding to adrenaline or isoproterenol was increased markedly at 24 h, reached a maximal level at 48-72 h and decreased subsequently. At 72 h after operation, β-adrenergic agonists-induced adenylate cyclase activity in partially hepatectomized rats were about 20-fold higher than those in sham-operated rats. Moreover, in liver plasma membranes, β-adrenergic response was increased about 2-fold after operation (Fig. 2).

Next, GTP analogs-induced adenylate cyclase activity in liver plasma membranes was compared between normal and partially hepatectomized rats (Fig. 2). The basal activity of adenylate cyclase was identical for both preparations from normal and partially hepatectomized rats. In the presence of 10 µM GTPγS or Gpp(NH)p, the activity was about two times higher in the latter than in the former, suggesting the induction of Gs during liver regeneration (Fig. 2). On the other hand, there was little difference in the forskolin-induced adenylate cyclase activity between both rats (Fig. 2). These results have confirmed previous reports that β-adrenergic response is enhanced by partial hepatectomy (11, 12), and that this enhancement is not accompanied by an alteration in the intrinsic activity of adenylate cyclase (12).

The Changes in the Amount of $G_{s\alpha}$ and $G_{i\alpha}$ after Partial Hepatectomy --- To monitor the changes in the amount of $G_{s\alpha}$ during liver regeneration of male rats, liver plasma membranes were treated with cholera toxin to introduce ADP-ribosyl moiety and subjected to SDS-PAGE. As shown in Fig. 3A, the amounts of 47 kDa and 42 kDa proteins (corresponding to $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$, respectively, specifically labeled by cholera toxin) were increased after partial hepatectomy, reached a maximal level at 48-72 h and subsequently decreased (Fig. 3B). The amounts of $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$ at 72 h after partial hepatectomy were

increased 2.4-fold and 1.8-fold, respectively, compared with those after sham-operation. These time courses were quite similar to that of the change in catecholamine-responsive adenylate cyclase activity (Fig. 1).

Next, to monitor the change in amount of $G_{i\alpha}$ during liver regeneration, membranes were treated with pertussis toxin to introduce ADP-ribosyl moiety and subjected to SDS-PAGE. As shown in Fig. 4A, 41 kDa protein, $G_{i\alpha}$ (18), were specifically labeled by pertussis toxin. The amount of $G_{i\alpha}$ increased after operation, reaching maximal level at 24 h and subsequently decreased (Fig. 4B). The amount of $G_{i\alpha}$ at 24 h after partial hepatectomy were increased 2.4-fold compared with that after sham-operation. The increment in the amount of $G_{i\alpha}$ was prior to that in β-adrenergic response (Fig. 1).

The Changes in the Level of $G_{s\alpha}$ mRNA after Partial Hepatectomy --- To ascertain whether the increment in the amounts of $G_{s\alpha}$ was caused by the elevated level of $G_{s\alpha}$ mRNA, the abundance of the two $G_{s\alpha}$ mRNAs was measured using oligonucleotides specific for $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$. Both probes hybridized to mRNAs of approximately 1800 nucleotides (Fig. 5A). This is in good agreement with the previously reported transcript sizes of the two forms of $G_{s\alpha}$ (25). As shown in Fig. 5B, the abundance of the two mRNAs increased after partial hepatectomy, reached maximal level at 24-48 h, and then decreased. The increases in the levels of the two mRNAs occurred slightly earlier than those in the amounts of the two $G_{s\alpha}$ proteins. The degree of increase in the level of $G_{s\alpha\text{-}L}$ mRNA was higher than that of $G_{s\alpha\text{-}S}$. This may reflect the differences in the increased amounts of the two proteins (Fig. 3).

Competitive Binding Study of β-Adrenergic Agonist in Liver Plasma Membranes --- To examine the effects of partial hepatectomy on the coupling state between β-adrenergic receptors and G_s, competitive experiments of an agonist with an antagonist were

carried out in liver plasma membranes from normal and partially hepatectomized male rat (Fig. 6). Inhibiting curves of [^{125}I]ICYP binding by isoproterenol in liver plasma membranes from normal rats were monophasic, and gave IC_{50} values of 580 ± 30 nM or 71 ± 5 nM in the presence or absence of GTPγS, respectively. Whereas the IC_{50} values in the presence of GTPγS were similar in partially hepatectomized rats and normal rats, the IC_{50} value of isoproterenol in the absence of GTPγS (18 ± 1 nM) was four times lower in the former than in the latter. Similar results were obtained when Gpp(NH)p was used instead of GTPγS (data not shown). The change of isoproterenol binding characters was reversible and diminished gradually: the IC_{50} values of isoproterenol for membranes prepared 9th day after partial hepatectomy were 390 ± 6 nM and 78 ± 1 nM in the presence and absence of GTPγS, respectively. The proportion of β-AR in the high affinity (R_H) and the low affinity (R_L) states was evaluated by Scatchard analysis (26) (Fig. 5, Table I). The proportion of the high affinity sites for isoproterenol in partially hepatectomized male rats was higher than that in normal male rats. The high-affinity (K_H) and the low-affinity (K_L) values of β-adrenergic receptors were, however, identical in both preparations. Since the high-affinity state of β-adrenergic receptors is produced by the receptor-G_s complex (27), these results indicated that the proportion of β-adrenergic receptors coupled with G_s increased in parallel with the enhancement of β-adrenergic responsiveness.

Binding Studies of β-Adrenergic Antagonists in Liver Plasma Membranes --- Binding study of [^{125}I]ICYP to liver plasma membranes was carried out to examine alterations in β-adrenergic receptors (Table I). Two days after partial hepatectomy, the number of β-adrenergic receptors in operated rats was about three times higher than that in normal rats, and this elevated level was significantly decreased by

9 days after operation. Sham operation caused no alterations in the binding parameters (data not shown), as reported previously by others (12).

Table II shows the concentrations required for the half-maximal inhibition of [^{125}I]ICYP binding by various β-adrenergic agonists. The order of the potencies of these β-adrenergic agonists to inhibit [^{125}I]ICYP binding was isoproterenol > adrenaline > salbutamol > noradrenaline in both preparations, indicating that the subclass of the β-adrenergic receptors was β_2-type in both case. Sham operation caused no alterations in the binding parameters (data not shown), as reported previously by others (12). These results has confirmed that the level of β-adrenergic receptors is elevated following partial hepatectomy (12), and that this elevation is not accompanied with characteristic alterations in the receptor (12).

Effects of Anti-Estrogen Agent on Adrenergic Agonists-Induced cAMP Accumulation in Hepatocytes --- To ascertain whether estrogen is involved in the acquisition of β-adrenergic function after partial hepatectomy, the effects of an anti-estrogen agent, tamoxifen, on catecholamine-sensitive adenylate cyclase activity was examined (Fig. 7). Effect of tamoxifen on the enzyme activity was not observed in sham-operated rats. In partially hepatectomized rats, administration of tamoxifen for 2 days after operation did not affect the basal level of cAMP accumulated in hepatocytes. However, this treatment caused a significant reduction in partial hepatectomy-induced emergence of β-adrenergic responsiveness. This result suggested that estrogen may be involved in the regulation of β-adrenergic function.

Effect of Adrenergic Agonist and Antagonist on Tryptic Digestion of G_s --- Trypsin treatment can be used for detecting conformational change of G proteins caused by the binding of guanine

nucleotides (14, 28-31). This method was applied to determine which form of G_s proteins couples to β-adrenergic receptors. Plasma membranes from partially hepatectomized male rats were preincubated in the presence or absence of Gpp(NH)p and/or isoproterenol, cleaved with trypsin, ADP-ribosylated by cholera toxin, and subjected to SDS-PAGE. Time course of tryptic digestion is shown in Figs. 8 and 9. The amino terminal domain of $G_{s\alpha}$ is necessary for ADP-ribosylation (28). The cleaved site of $G_{s\alpha}$ by trypsin locates within that domain (29). Thus, cholera toxin cannot label digested $G_{s\alpha}$. In the absence of Gpp(NH)p, $G_{s\alpha\text{-}L}$ was gradually trimmed by trypsin, whereas $G_{s\alpha\text{-}S}$ was essentially insensitive. Preincubation with 100 μM Gpp(NH)p markedly increased in the sensitivities of both proteins to trypsin. In contrast, this effect was significantly retarded by GDP (Table III). These results suggest that the Gpp(NH)p-bound forms of both $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$ were more sensitive to trypsin than their GDP-bound forms. When preincubated in the presence of 100 nM Gpp(NH)p and 10 μM isoproterenol, the trypsin-sensitivity of $G_{s\alpha\text{-}L}$ was increased compared with that following preincubation with 100 nM Gpp(NH)p alone (Figs. 8 and 9). On the other hand, addition of isoproterenol to the preincubation mixture containing 100 nM Gpp(NH)p had no effect on $G_{s\alpha\text{-}S}$. The effect of isoproterenol on $G_{s\alpha\text{-}L}$ was inhibited by the addition of the antagonist propranolol in the preincubation mixture (Table III). Similar results were obtained when adrenaline was used instead of isoproterenol (data not shown). These results suggested that β-adrenergic receptors catalyze the exchange of GDP for GTP on $G_{s\alpha\text{-}L}$ more efficiently than that on $G_{s\alpha\text{-}S}$.

DISCUSSION

The results of the present study show that significant increases in the level of $G_{s\alpha}$ protein (Fig. 3) and mRNA (Fig. 5) are closely correlated with the acquisition of β-adrenergic response after partial hepatectomy (Figs. 1 and 2) . Furthermore, the ratio of $G_{s\alpha\text{-}L}$ to $G_{s\alpha\text{-}S}$ was increased responding to an alteration in β-adrenergic response during liver regeneration (Fig. 3). In addition, the earlier results by others (11-13) has been confirmed in this study. The appearance of β-adrenergic response in the initial stage of liver regeneration was accompanied with an elevated level of β-adrenergic receptors (Table II). Furthermore, the type of β-adrenergic receptors were exclusively β_2-type in adult male rat livers, and not altered throughout regeneration (Table III). Finally, the intrinsic activity of adenylate cyclase was unaffected by partial hepatectomy (Fig. 2). Thus, up-regulations of β-adrenergic receptors and $G_{s\alpha}$ appear to lead the enhanced coupling of receptors with G proteins (Fig. 6 and Table I).

There was a possibility that the attenuation of G_i-mediated inhibition of adenylate cyclase is involved in the development of β-adrenergic receptor-mediated adenylate cyclase activation. This possibility, however, was not supported for the following reasons. (A) Hepatic adenylate cyclase activity responding to isoproterenol was also higher in partially hepatectomized rats than that in normal (Fig. 1). (B) Adrenaline-induced cAMP accumulation in hepatocytes was increased by partial hepatectomy even in the presence of phentolamine, an α-adrenergic antagonist (data not shown). (C) The amount of $G_{i\alpha}$ was not decreased, but increased in the initial stage of the regenerating period (Fig. 4). Since G_i can play a direct role in the initiation of DNA synthesis (32), G_i may be involved in the hepatocyte proliferation observed following partial hepatectomy.

Rat liver function and composition display sexual dimorphisms and are markedly dependent on sex steroids (8-10). Both hepatic contents of estrogen and androgen receptors were altered dramatically by partial hepatectomy (8, 10). Total hepatic content and nuclear retention of estrogen receptors and serum estradiol increase in partially hepatectomized males rats (8, 10). On the other hand, those of androgen receptors and serum testosterone level demonstrate a massive decline after partial hepatectomy (10). Moreover, estrogen antagonist inhibits proliferation of hepatocytes following partial hepatectomy (33). Thus, estrogen, but not androgen, may have an important role in the process of liver regeneration (10, 33). The hormone can also be involved in the sex-dependent emergence of β-adrenergic agonists-sensitive adenylate cyclase activity (Chapter II). Taken together, it is possible that estrogen may be involved in the emergence of β-adrenergic function following partial hepatectomy. Indeed, the enhancement of β-adrenergic function during liver regeneration was attenuated by anti-estrogen treatment (Fig. 7). Thus, estrogen appears to play an pivotal role in the appearance of β-adrenergic function.

Receptor-G protein interaction can be monitored by determining the receptor-mediated stimulation of [γ-^{35}S]GTPγS binding (34) and GTPase activity (35). However, these activities responding to agonists could not be detected in liver plasma membranes. This failure may be due to the presence of other G proteins such as G_i (18). To overcome this problem, a method using tryptic digestion was applied. This method was developed from studies on transducin (G_t) (14). G_t is known to activate cGMP phosphodiesterase in response to photolyzed rhodopsin (36). The free α subunit of transducin ($G_{t\alpha}$) bound with GTPγS is digested by trypsin more rapidly than the trimeric $G_{t\alpha}$ bound with GDP at the amino- and carboxyl-terminal of $G_{t\alpha}$ (14). In the case of $G_{s\alpha}$, the GTP analog-bound

peptide is also resistant to trypsin at Arg^{232} which corresponds to Arg^{204} of $G_{t\alpha}$ (29). This site, in its inactive GDP-bound form, is cleaved by trypsin (29). Therefore, it is possible that GTP analogs affect the sensitivity of $G_{s\alpha}$ to trypsin in the initial stage of this proteolysis. Indeed, trypsinization of $G_{s\alpha}$ in plasma membranes from partially hepatectomized male rat livers was promoted by Gpp(NH)p in a concentration-dependent manner (Figs. 8 and 9). This effect of Gpp(NH)p was abolished in the presence of GDP (Table IV). Therefore, the acceleration of proteolysis of $G_{s\alpha}$ reflects the conversion of GDP-bound $G_{s\alpha}$ to the GTP-bound form. $G_{s\alpha\text{-}L}$ was more susceptible to this protease in the presence of β-adrenergic agonists (Figs. 8 and 9), and the effects of agonists were abrogated in the presence of β-adrenergic antagonist (Table IV). On the other hand, the sensitivity of $G_{s\alpha\text{-}S}$ to tryptic cleavage remained unchanged regardless of the presence or absence of the agonist (Figs. 8 and 9) and/or the antagonist (Table IV). These findings indicate that a β-adrenergic agonist-receptor complex catalyzes the exchange from GDP to GTP on $G_{s\alpha\text{-}L}$, but not on $G_{s\alpha\text{-}S}$.

The present results demonstrate that the partial hepatectomy-dependent appearance of β-adrenergic function can be ascribed to increases in the number of β-adrenergic receptors, the amount of $G_{s\alpha}$ and the interaction between the receptors and Gs. Furthermore, β-adrenergic receptors appear to be more efficiently coupled with $G_{s\alpha\text{-}L}$ than $G_{s\alpha\text{-}S}$. Moreover, it seems likely that estrogen is involved in this appearance of β-adrenergic function.

Table I

Kinetic parameters for specific isoproterenol binding to liver plasma membranes

	Day 0	Day 2	Day 9
K_L (nM)	330 ± 18	450 ± 23	192 ± 25
K_H (nM)	5.2 ± 1.1	6.3 ± 1.5	5.1 ± 0.8
K_L/K_H	68 ± 5	73 ± 4	40 ± 3
R_L (%)	55 ± 5	24 ± 4*	68 ± 5
R_H (%)	45 ± 6	76 ± 5*	32 ± 7

Membranes were prepared on 0, 2nd or 9th day after partial hepatectomy. Competition experiments were carried out as outlined in Fig. 6. The parameters were calculated by Scatchard analysis of the data in Fig. 6. Values are the mean ± S.E. of 4 separate experiments. *P<0.05 *versus* to Day 0 from t test.

Table II

Kinetic parameters for specific [^{125}I]ICYP binding to liver plasma membranes

Days after operation	B max (fmol / mg protein)	K_d (pM)
Day 0	72.0 ± 5.4	129 ± 25
Day 2	244.8 ± 22.1**	91 ± 15
Day 9	169.6 ± 22.4*	93 ± 10

Liver plasma membranes were prepared on 0, 2nd 9th day after partial hepatectomy. Bmax and K_d values were determined from Scatchard analysis of [^{125}I]ICYP binding data. Values are the mean ± S.E. of 4 separate experiments. *P<0.05 or **P<0.01 *versus* to Day 0 form t test.

Table III

Inhibition of [^{125}I]ICYP binding to plasma membranes by adrenergic agonists

	IC50 (μM)	
	Nomal	Partial Hepatectomy
Isoproterenol	0.071 ± 0.005	0.018 ± 0.001
Adrenaline	1.8 ± 0.1	0.43 ± 0.02
Salbutamol	2.0 ± 0.2	0.8 ± 0.01
Noradrenaline	3.9 ± 0.1	2.2 ± 0.1

Liver plasma membranes prepared from 0 or 2nd day after partial hepatectomy were incubated with 100 pM [^{125}I]ICYP and various concentrations of adrenergic agonists. The data are IC_{50} values of [^{125}I]ICYP binding. Values are the mean ± S.E. from 4 separate experiments.

Table IV

The effect of guanine nucleotides and adrenergic ligands on proteolysis of $G_{s\alpha}$ by TPCK-trypsin.

	47 kDa (%)	42 kDa (%)
(A) no addition	81 ± 3	100 ± 5
10 μM Gpp(NH)p	40 ± 3 a)	60 ± 4a)
10 μM Gpp(NH)p + 100 μM GDP	55 ± 4b)	81 ± 3c)
(B)100 nM Gpp(NH)p	71 ± 2	86 ± 2
100 nM Gpp(NH)p + 10 μM Isoproterenol	51 ± 1d)	90 ± 3
100 nM Gpp(NH)p + 10 μM Isoproterenol + 10 μM Propranolol	69 ± 3	83 ± 4

Membranes prepared on 2nd day after partial hepatectomy were preincubated with the indicated concentrations of various ligands for 20 min at 30 °C. After trypsin treatment for 2 min (A) or 1 min (B) at 30 °C, samples were subjected to ADP-ribosylation. Control values were detected in the absence of trypsin. Values are the mean ± S.E. for 9 separate experiments. a) $P < 0.01$ *versus* no addition, b) $P < 0.05$ *versus* to 10 μM Gpp(NH)p, c) $P < 0.01$ *versus* to10 μM Gpp(N)p and d) $P < 0.01$ *versus* to 100 nM Gpp(NH)p from t test.

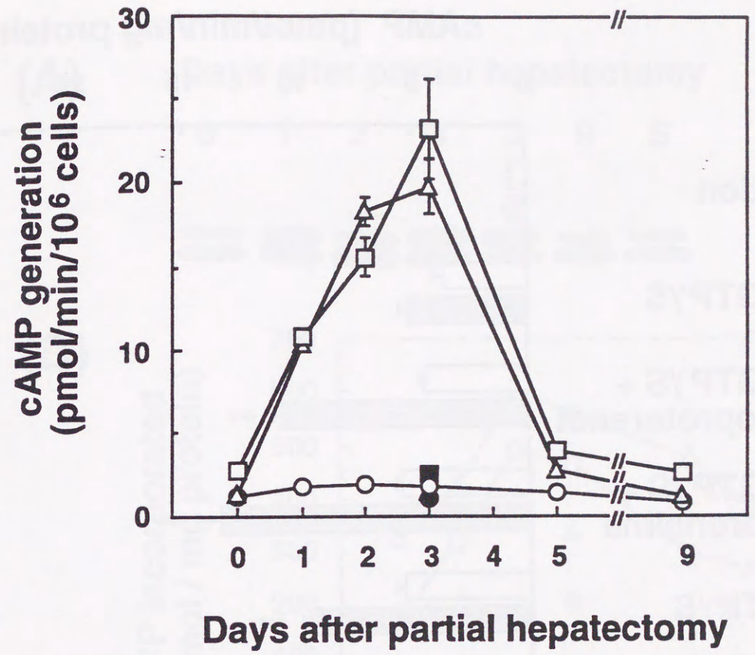

Fig. 1. **The changes in adrenergic agonists-induced cAMP accumulation in hepatocytes after partial hepatectomy.** Hepatocytes prepared from partially hepatectomized (○, △, □) or sham-operated (●, ▲, ■) rats were incubated with 10 μM isoproterenol (△, ▲) or 10 μM adrenaline (□, ■), or without addition (○, ●). Values are the mean ± S.E. for 4 separate experiments.

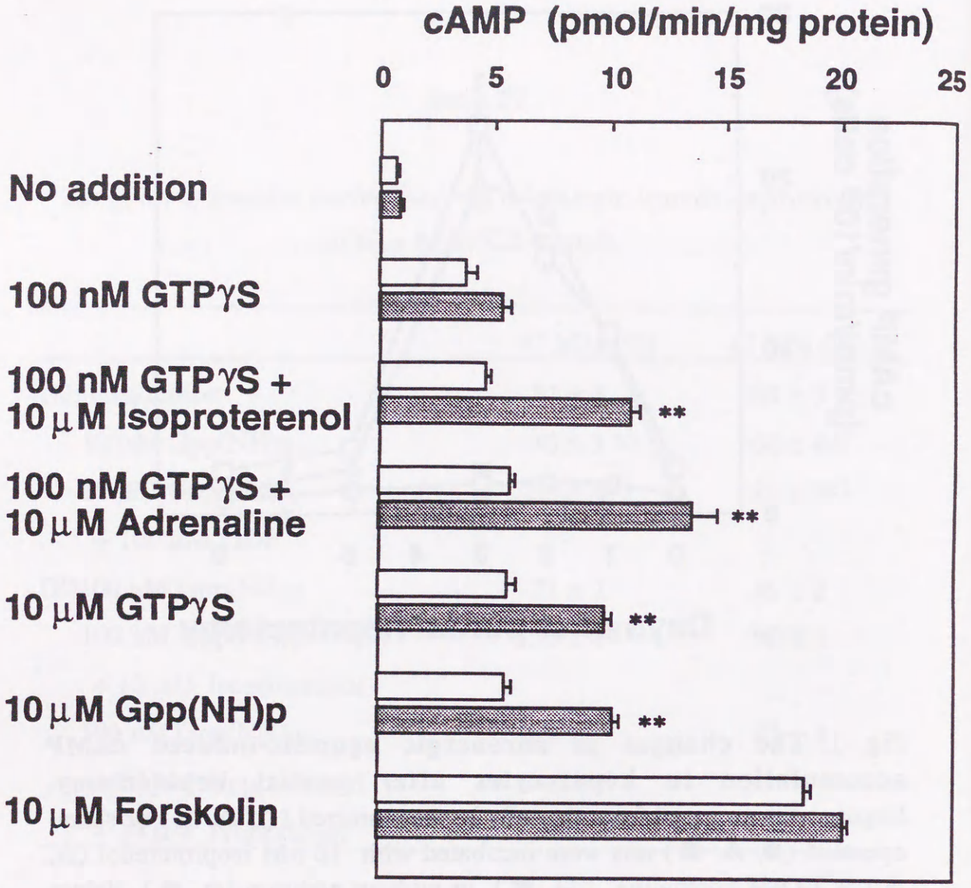

Fig. 2. **Effect of partial hepatectomy on adenylate cyclase activity in liver plasma membranes.** Liver plasma membranes prepared on 0 (□) or 2nd day (■) after partial hepatectomy were incubated with the indicated concentrations of various stimuli. Values are the mean ± S.E. for 4 separate experiments. **P<0.01 *versus* to Day 0.

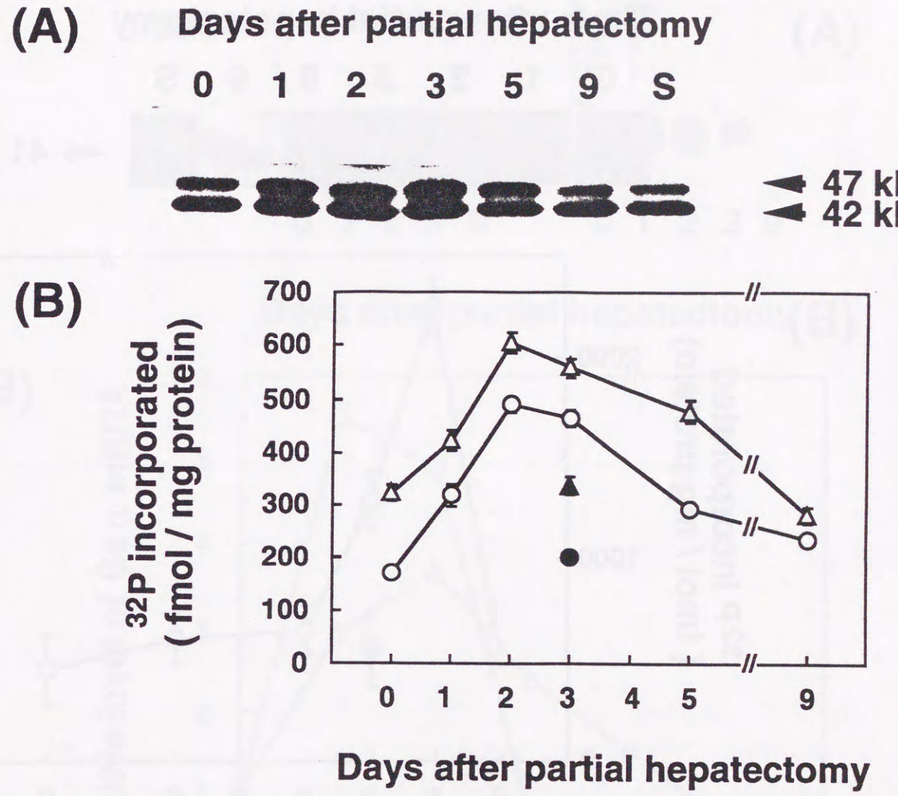

Fig. 3. **Time course of the changes in the amount of $G_{s\alpha}$ and after partial hepatectomy.** Autoradiogram (A) and quantification (B) of [^{32}P]ADP-ribosylated $G_{s\alpha}$ proteins. Membranes were prepared from partially hepatectomized (○, △) or sham-operated (●, ▲; S) rats. The incorporation of ^{32}P into 47 kDa $G_{s\alpha\text{-}L}$ (○, ●) and 42 kDa $G_{s\alpha\text{-}S}$ (△, ▲) was measured as described under "Experimental Procedures". Values are the mean ± S.E. for 4 separate experiments.

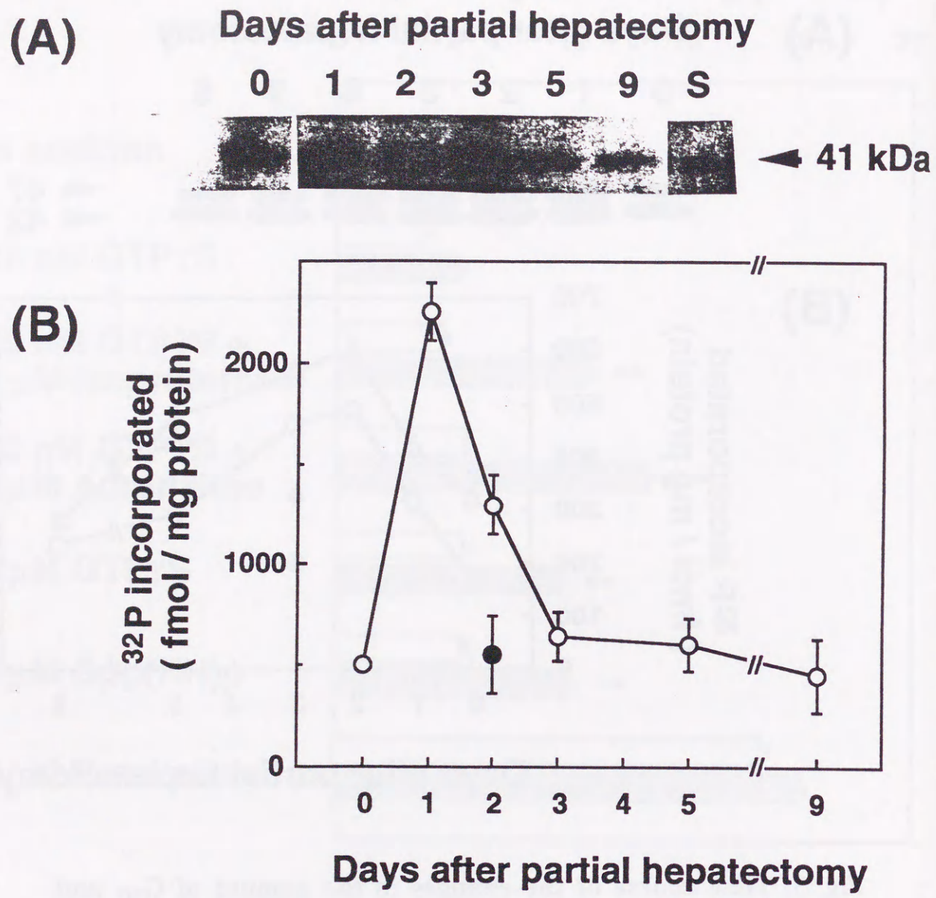

Fig. 4. **The changes in the amount of $G_{i\alpha}$ and after partial hepatectomy.** Autoradiogram (A) and quantification (B) of [^{32}P]ADP-ribosylated $G_{i\alpha}$ proteins. Membranes were prepared from partially hepatectomized (○) or sham-operated (●) rats. The incorporation of ^{32}P in 41 kDa $G_{i\alpha}$ was measured as described under "Experimental Procedures". Values are the mean ± S.E. for four separate experiments.

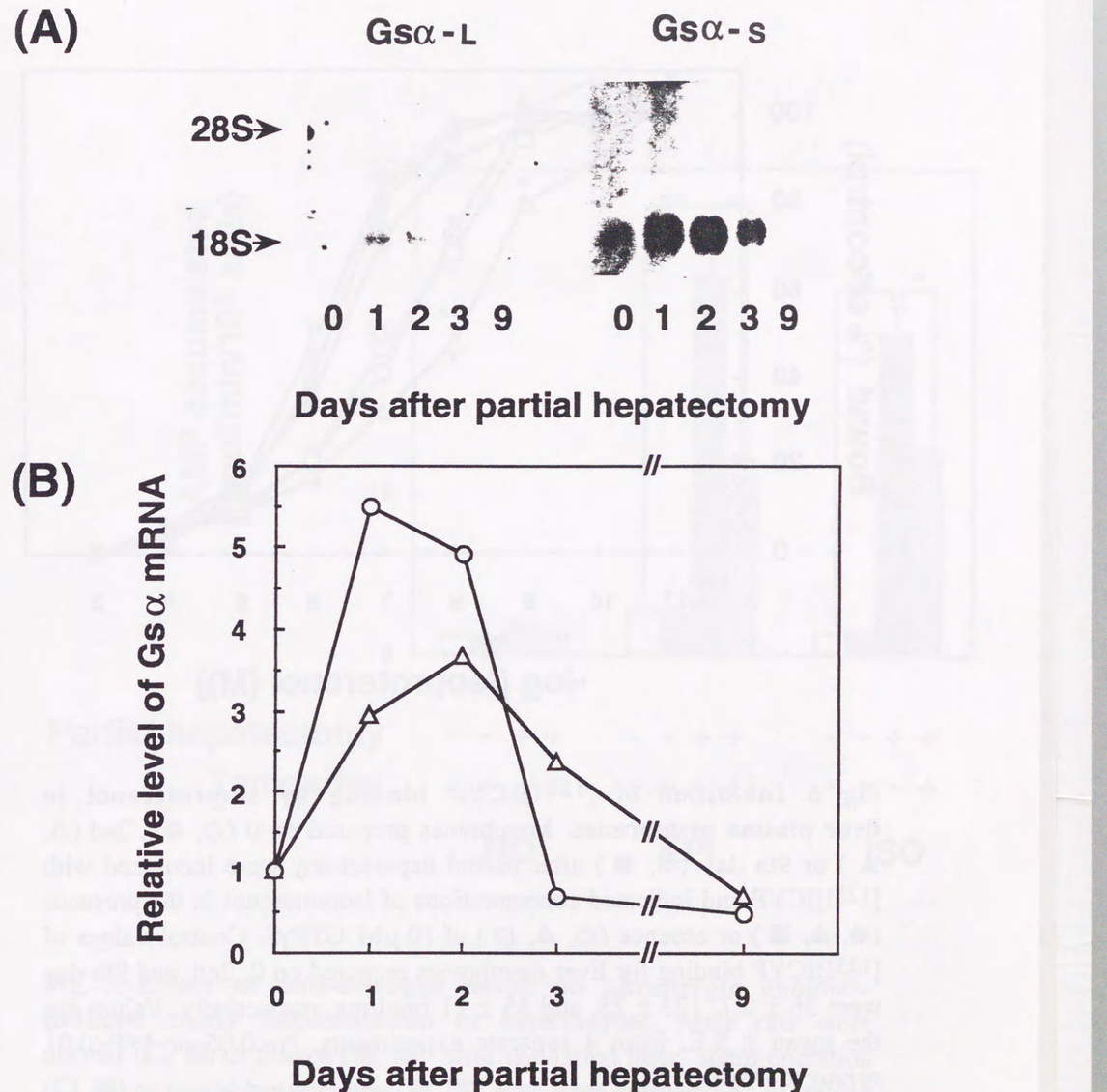

Fig. 5. **The changes in the level of $G_{s\alpha}$ mRNA after partial hepatectomy.** (A) Hybridization of poly(A)$^+$ RNA from partially hepatectomized rats with oligonucleotides specific for $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$. 18S and 28S rRNAs were used as markers. Their positions were indicated by arrows. (B) Changes in the relative levels of the mRNAs of $G_{s\alpha\text{-}L}$ (○) and $G_{s\alpha\text{-}S}$ (Δ). Values are expressed as the relative ratio over the level before partial hepatectomy.

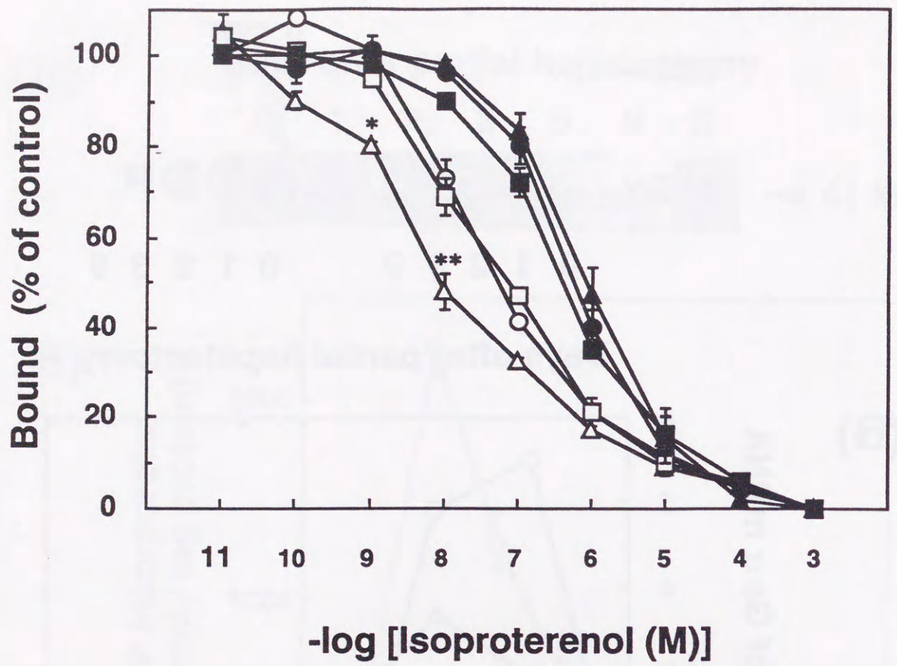

Fig. 6. **Inhibition of [^{125}I]ICYP binding by isoproterenol in liver plasma membranes.** Membranes prepared on 0 (○, ●), 2nd (△, ▲) or 9th day (□, ■) after partial hepatectomy were incubated with [^{125}I]ICYP and indicated concentrations of isoproterenol in the presence (●, ▲, ■) or absence (○, △, □) of 10 μM GTPγS. Control values of [^{125}I]ICYP binding for liver membranes prepared on 0, 2nd, and 9th day were 36 ± 2.7, 123 ± 25, and 85 ± 11 fmol/mg, respectively. Values are the mean ± S.E. from 4 separate experiments. *p<0.05 or **P<0.01 *versus* to Day 0.

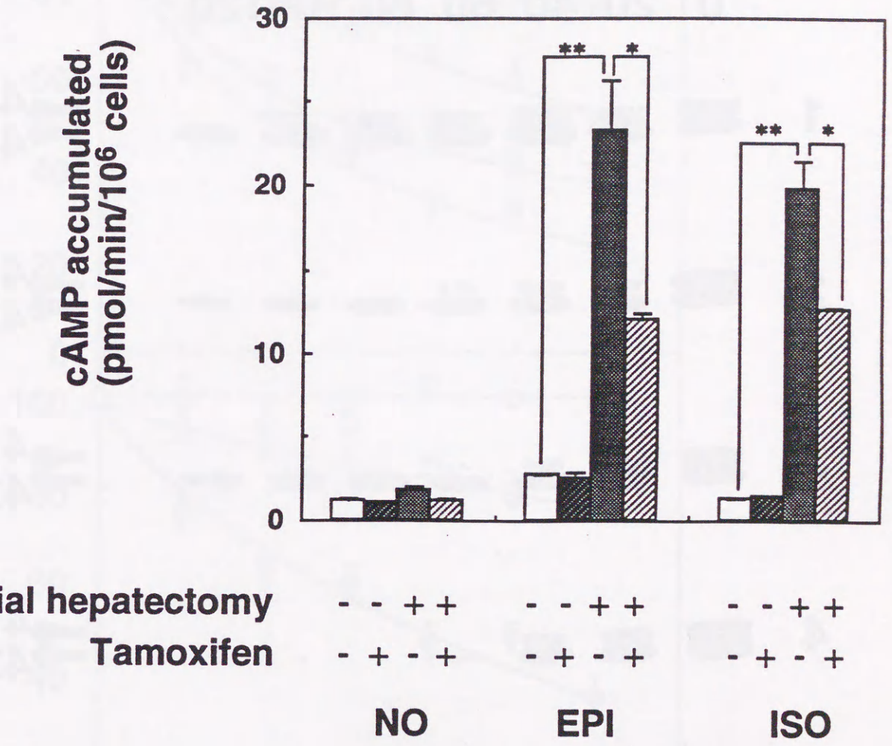

Fig. 7. **Effect of anti-estrogen agent on adrenergic agonists-induced cAMP accumulation in hepatocytes.** Male rats were normal (□, ■) or treated (▩, ▨) with tamoxifen after sham-operation (□, ▩) or partial hepatectomy (■, ▨). Rats were untreated (-) or treated (+) by partial hepatectomy and/or tamoxifen. Hepatocytes were incubated with 10 μM adrenaline (EPI), 10 μM isoproterenol (ISO) or without addition (NO). Values are the mean ± S.E. of 4 separate experiments. *P<0.05 *versus* to tamoxifen treated rats, t test. **P<0.01 *versus* to sham-operated rats, t test.

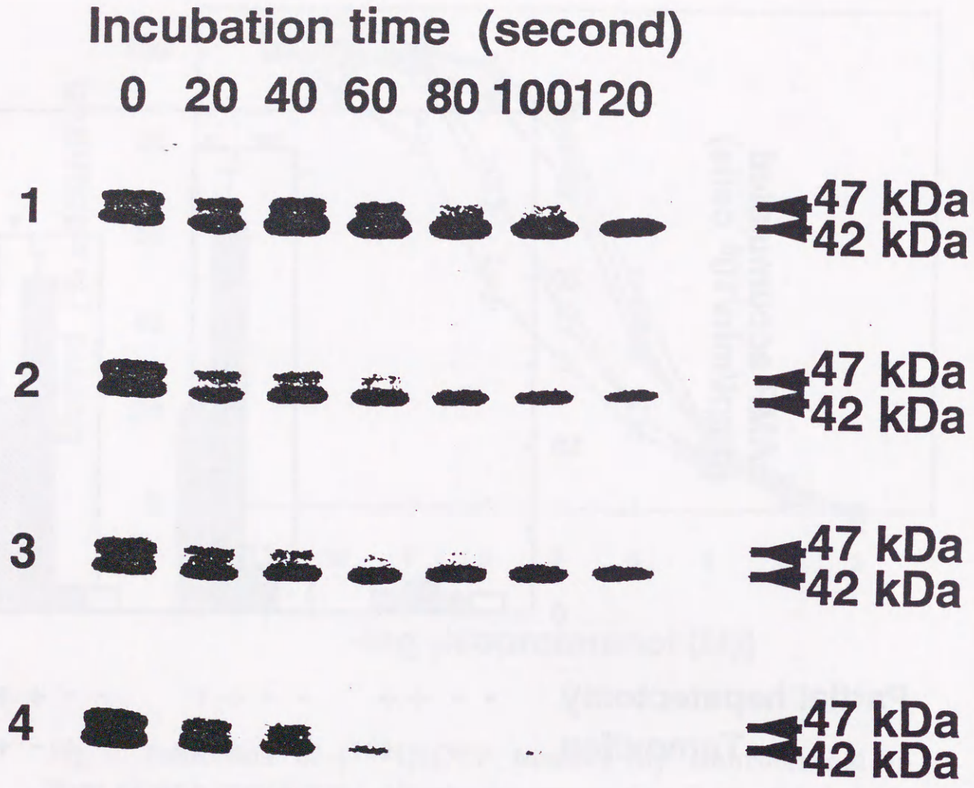

Fig. 8. **Autoradiogram of [^{32}P]ADP-ribosylated $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$ after TPCK-trypsin treatment.** Liver plasma membranes prepared on 2nd day after partial hepatectomy were preincubated with 100 nM Gpp(NH)p (lane 2), 100 nM Gpp(NH)p + 10 μM isoproterenol (lane 3) for 20 min or 100 μM Gpp(NH)p for 60 min (lane 4), or without addition (lane 1). Membranes were then incubated with trypsin, treated with cholera toxin to introduce ADP-ribosyl moiety, and subjected to SDS-PAGE.

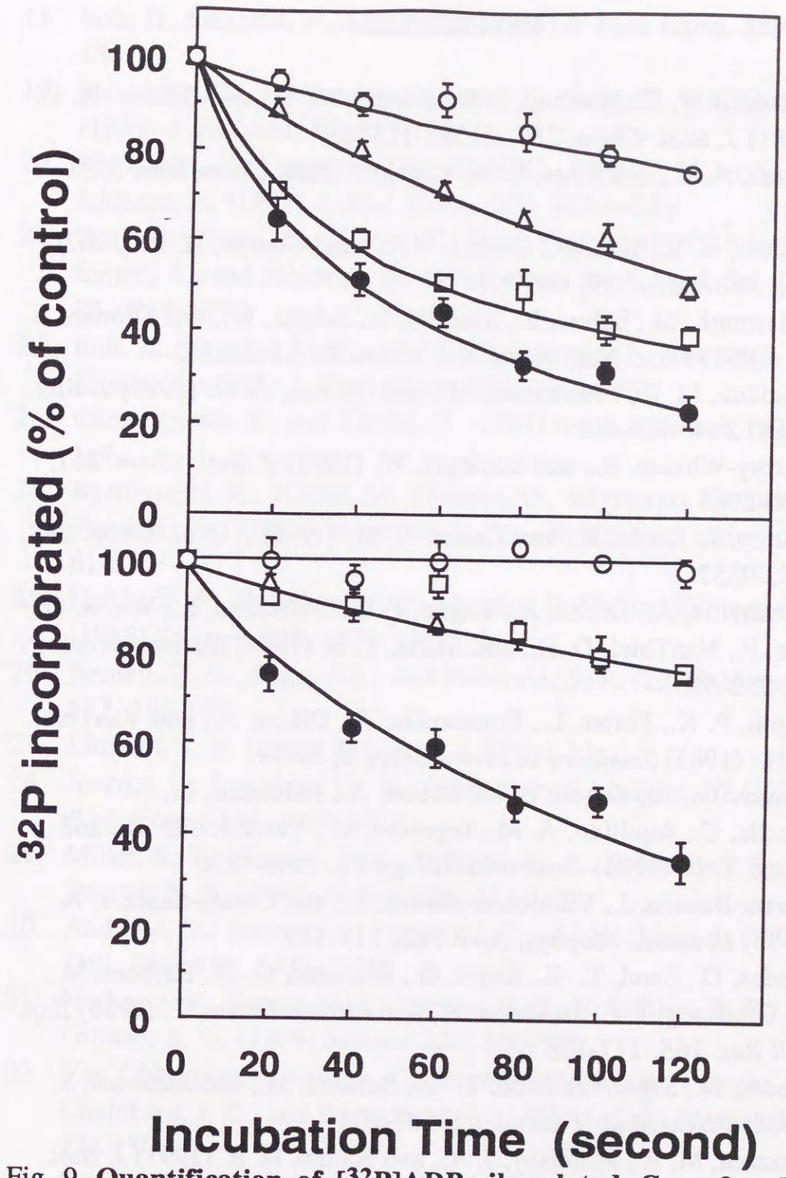

Fig. 9. **Quantification of [^{32}P]ADP-ribosylated $G_{s\alpha}$ after TPCK-trypsin treatment.** Tryptic digestion of $G_{s\alpha\text{-}L}$ (top panel) and $G_{s\alpha\text{-}S}$ (bottom panel) was carried out as outlined in Fig 8. Membranes were preincubated with 100 nM Gpp(NH)p (△), 100 nM Gpp(NH)p + 10 μM isoproterenol (□) for 20 min or 100 μM Gpp(NH)p for 60 min (●), or without addition (○). Control values were detected in the absence of trypsin. Values are the mean ± S.E. for 9 separate experiments.

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Chapter IV

Coupling of Glucagon Receptors with Stimulatory G protein in Partially Hepatectomized Rat Liver

INTRODUCTION

Liver plays a central role in blood glucose homeostasis through gluconeogenesis and glycogenolysis. In two enzymatic pathways, glucagon and adrenaline are the key hormones. These actions of glucagon on the liver result when this hormone increases the intracellular cAMP *via* glucagon receptors (1), and G_s is the G protein involved in this pathway (1). However, whether glucagon and β-adrenergic receptors share a common cAMP-generating pathway remains controversial.

Hepatic adenylate cyclase activities responding to glucagon and catecholamine are very high in young rats (2, 3). During development, however, both responses decrease markedly and reach adult levels at 60 days after birth. In adult male rat livers, the response to glucagon is still considerable, whereas that to catecholamine is very low (2, 3). In contrast, hepatic phospholipase C activity responding to adrenaline increases markedly, and adrenaline-induced glucose metabolism is mediated mainly by α_1-adrenergic receptors (3). However, the predominant subtype of adrenergic receptors contributing to the regulation of liver function is converted from an α_1-type to a β-type after partial hepatectomy (4, 5). The reciprocal expression patterns of α_1- and β-adrenergic receptors contribute to this conversion (5, 6). The number of glucagon receptors, on the other hand, was found to be reduced following partial hepatectomy (7, 8). Nevertheless, partial hepatectomy did not affect the level of glucagon-induced adenylate cyclase activity (5). As a result of these two conflicting phenomena, glucagon-induced cAMP generation in partially hepatectomized male rat livers was reinvestigated in this study.

With a method using tryptic digestion to evaluate the coupling of receptors with G proteins, $G_{s\alpha\text{-}L}$ was identified as the preferred G

protein for β-adrenergic receptors in partially hepatectomized male rat livers (Chapter III). Besides $G_{s\alpha\text{-}L}$, $G_{s\alpha\text{-}S}$ is another isoform of $G_{s\alpha}$ (1). The effector-activating residues of $G_{s\alpha}$ are common to both $G_{s\alpha}$ isoforms (9), indicating that $G_{s\alpha\text{-}S}$ is capable of coupling with the receptors which stimulate adenylate cyclase. Since the enzyme activities induced by glucagon and catecholamine are separable and additive in liver homogenates from adult male rats, the hepatic adenylate cyclase system responding to glucagon differs from that responding to catecholamines (10, 11). It is then possible that $G_{s\alpha\text{-}S}$ preferentially mediates the stimulation of the enzyme by glucagon. In this study, the radio binding assay and the tryptic digestion method were used to analyze coupling of glucagon and β-adrenergic receptors with G_s in partially hepatectomized male rat livers. The primary question is whether glucagon receptors share common $G_{s\alpha\text{-}L}$ with β-adrenergic receptors or take independent pathways through $G_{s\alpha\text{-}S}$.

EXPERIMENTAL PROCEDURES

Materials --- [*Adenylate* α-^{32}P]NAD (29.6 TBq/mmol), [^{125}I]glucagon (81.4 TBq/mmol) and [^{125}I]ICYP (8.14 TBq/mmol) were purchased from Du Pont-New England Nuclear. Adrenaline bitartrate, isoproterenol bitartrate, glucagon, cholera toxin, bovine serum albumin, IBMX, TPCK-treated trypsin and soybean trypsin inhibitor were obtained from Sigma Chemical Co. GTPγS and Gpp(NH)p were from Boehringer Mannheim. X-ray film (New-XR), film cassettes and intensifying screens were from Fuji Photo Film. The assay kit for cAMP was from Yamasa Shoyu Co. All other chemicals were reagent grade and obtained from commercial sources.

Animals and Operations --- Male Slc:Wistar strain rats (9-10 weeks old), weighing between 230-260 g, were obtained from Japan SLC, Inc. Partial hepatectomy (70%) was performed according to the method of Higgins *et al.* (12) as described in Chapter III. All surgical procedures were performed between 8:00 a.m. and noon.

Measurement of Adenylate Cyclase Activity --- Rat hepatocytes and liver plasma membranes were prepared by the collagenase perfusion method of Tanaka *et al.* (13) and the percoll-centrifugation method of Lynch *et al.* (14), respectively, as described in Chapter II. Cyclic AMP in hepatocytes and liver plasma membranes was measured according to the method of Itoh *et al.* (15) as described in Chapter II.

Measurement of $[Ca^{2+}]_i$ in Hepatocytes --- $[Ca^{2+}]_i$ was measured with the fluorescent Ca^{2+} indicator fura-2, as previously reported by Grynkiewicz *et al.* (16). Isolated hepatocytes were preincubated with 5 μM fura-2 acetoxymethylester (fura-2 AM added from a 1 mM stock solution in dimethylsulfoxide) for 15 min at 37 °C under an atmosphere of 95 % O_2 - 5 % CO_2. The cells were then washed by centrifugation at 50 x g for 1 min and resuspended to 3 x 10^6 cells / ml in a fresh Hepes buffer medium consisting of 10 mM Hepes (pH 7.4), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$ and 5 mM $NaHCO_3$. The cells were washed twice and finally resuspended in the same fresh buffer. After incubation of the cells for additional 5 min at 37 °C, fluorescence intensities were measured at 510 nM with two excitation wavelengths, 340 nm and 380 nm, using a CAF-100 Ca^{2+} analyzer equipped with a magnetic stirrer and a thermostated cell holder, and the intensity ratio of 340 nM to 380 nm in the excitation wavelength was calculated.

Binding Studies of [^{125}I]ICYP and [^{125}I]Glucagon in Liver Plasma Membranes --- The binding study of [^{125}I]ICYP was performed according to the method of Nakamura *et al.* (3) as described in Chapter II. The binding study of [^{125}I]ICYP was carried out by a modification of the method of Rodbell *et al.* (17). The reaction mixture consisted of 50 mM Tris-HCl (pH 7.4), 2.8 % bovine serum albumin (Fraction V), 1 mM EDTA, 300 pM [^{125}I]glucagon (approximately 100,000 dpm), and liver membranes (10 μg of protein). 100 μl of reaction mixture was incubated at 30 °C for 45 min. The reaction was terminated by adding 2.5 ml ice cold 50 mM Tris-HCl (pH 7.4), immediately followed by rapid filtration through Whatman GF/C glass fiber filters (presoaked in 1 % polyethleneimine) under reduced pressure. The filters were then quickly washed five times with 2.5 ml ice-cold 50 mM Tris-HCl (pH 7.4). Specific binding was calculated by subtracting the nonspecific binding in the presence of 1 μM glucagon and was about 80 % of the total binding.

ADP-Ribosylation and Tryptic Digestion of Liver Plasma Membranes --- The procedure for ADP-ribosylation by cholera toxin was according to the method of Nakamura *et al.* (18) as described in Chapter II. Trypsin treatment of liver plasma membranes was performed according to the method of Mazzoni *et al.* (19) as described in Chapter III.

RESUTS

The changes in Generation of cAMP and Elevation of $[Ca^{2+}]_i$ in Rat Livers after Partial Hepatectomy ---

The changes in glucagon- and adrenaline-sensitive adenylate cyclase activity after partial hepatectomy are shown in Fig. 1. Analogous to the

increment in β-adrenergic response, glucagon-induced accumulation of cAMP in hepatocytes increased progressively after operation, achieving a maximal value at about 48 h, and then subsequently declining. At 48 h after surgery, glucagon-sensitive adenylate cyclase activity was increased about 2-fold. On the other hand, the increment in the enzyme activity was not observed in sham-operated rats. In addition, generation of cAMP in response to glucagon was also enhanced in liver plasma membranes during liver regeneration (Fig. 2). In contrast, adrenaline-induced elevation of $[Ca^{2+}]_i$ was lowered in fura-2 AM-loaded hepatocytes after operation (Fig. 3). Pharmacological studies using agonists and antagonists indicated that elevation of $[Ca^{2+}]_i$ responding to adrenaline was mediated *via* α_1-adrenergic receptors (data not shown). Thus, it has been confirmed that adrenergic function is converted from α_1-type to β-type during liver regeneration (4, 5). Furthermore, it was indicated that hepatic adenylate cyclase activity in response to glucagon was clearly increased following partial hepatectomy.

Binding Studies of [^{125}I]Glucagon in Liver Plasma Membranes --- In order to examine alterations in glucagon receptors following partial hepatectomy, binding parameters for [^{125}I]glucagon to liver plasma membranes were calculated by Scatchard analysis (Table I). It has been confirmed that the number of β-adrenergic receptors is increased after surgery (5, 6). However, Bmax and K_d values of glucagon receptors were not changed after operation.

Effects of Glucagon and β-Adrenergic Agonists on Hepatic Adenylate Cyclase Activity --- To ascertain whether the effects of glucagon and catecholamines on hepatic adenylate cyclase activity are additive, glucagon- and adrenaline-induced cAMP accumulation in rat hepatocytes were examined (Fig. 4). In both normal and partially hepatectomized male rat livers, glucagon increased cAMP accumulation

in a concentration-dependent manner (Fig. 4). When adrenaline was added at the concentration at which hepatic adenylate cyclase activity is maximally stimulated (10 μM), the activity of adenylate cyclase responding to glucagon was enhanced, but the maximal adenylate cyclase activity remained unaffected.

As shown in Fig. 5, adenylate cyclase activities were examined in liver plasma membranes from partially hepatectomized male. In the presence of nonhydrolyzable GTP analogs (100 nM), adenylate cyclase activities responding to glucagon (1 μM) and isoproterenol (10 μM) showed 4-fold and 1.5-fold increases, respectively. The concentrations of these agonists were sufficient to exert their maximal effects on their hepatic adenylate cyclase activity. When the two hormones were added together, the enzyme activity was nearly the same as that induced by glucagon alone. Replacement of isoproterenol by adrenaline gave the same results (data not shown). Clearly, the effects produced by glucagon and β-adrenergic agonists were not additive.

Effects of Glucagon on β-Adrenergic Agonists Binding to β-Adrenergic Receptors --- To test the possibility that glucagon may influence the coupling of β-adrenergic receptors with G_s, the effect of glucagon on inhibition of [^{125}I]ICYP binding by isoproterenol was examined in liver plasma membranes from partially hepatectomized male rats (Table II, Figs. 6 and 7). Isoproterenol inhibited the binding of [^{125}I]ICYP with β-adrenergic receptors in a concentration-dependent manner (Fig. 6). The IC_{50} of isoproterenol for [^{125}I]ICYP binding was 18 ± 1 nM. This competition curve was best fitted by a two-site model, indicating the interaction of isoproterenol with the low- and high-affinity states of β-adrenergic receptors (Table II). The complex of β-adrenergic receptors and GDP-bound G_s exhibited high affinity for agonists, whereas the receptor alone showed low affinity (20, 21). In the presence

of GTPγS (10 μM), the competition curves had a Hill coefficient of ~1.0, suggesting a single site with low affinity (K_i = 513 ± 21 nM).

In the absence of GTPγS, the binding of [^{125}I]ICYP and isoproterenol to β-adrenergic receptors was not affected by glucagon (data not shown), whereas, in the presence of GTPγS (10 nM), glucagon decreased the apparent affinity of β-adrenergic receptors for isoproterenol (Fig. 6). In the presence of 10 nM GTPγS, the IC_{50} of isoproterenol for [^{125}I]ICYP binding was 44 ± 5 nM. Glucagon (1 μM) increased the IC_{50} to 158 ± 13 nM. The effect of glucagon on isoproterenol binding to β-adrenergic receptors was observed in the presence of 1-30 nM GTPγS (Fig. 7). Scatchard analysis (22) showed that glucagon decreased the fraction of β-adrenergic receptors in the high affinity state (percentage of R_H) without altering the value of high-affinity (K_H) (Table II). Similar results were obtained when adrenaline and Gpp(NH)p were used instead of isoproterenol and GTPγS, respectively (data not shown). Glucagon appears to uncouple β-adrenergic receptors from G_s in the presence of GTP.

Effects of β-Adrenergic Agonists on Glucagon Binding to Glucagon Receptors --- Since glucagon attenuates the accessibility of β-agonists to β-adrenergic receptors, β-adrenergic agonists might also decrease the binding of glucagon to its receptors. To test the possibility, the specific binding of [^{125}I]glucagon to liver plasma membranes was examined with GTPγS at various concentrations in the presence or absence of isoproterenol. The binding parameters of glucagon to glucagon receptors were not affected by isoproterenol (data not shown). As reported previously (23), GTPγS reduced the binding of [^{125}I]glucagon in a concentration-dependent manner (Fig. 5). Contrary to expectation, 10 μM isoproterenol did not affect the affinity of glucagon receptors for glucagon in the presence of GTPγS. Similar results were

obtained when isoproterenol and GTPγS were replaced by adrenaline and Gpp(NH)p, respectively (data not shown).

Effect of Glucagon on Tryptic Digestion of G_s --- To determine which form of G_s is coupled to glucagon receptors, the effect of glucagon on the trypsin-sensitivity of G_s was investigated. Liver plasma membranes from partially hepatectomized male rats were preincubated with glucagon or isoproterenol in the presence of Gpp(NH)p, cleaved with trypsin, treated with cholera toxin to introduce ADP-ribosyl moiety, and subjected to SDS-PAGE. Figs. 8 - 11 show the autoradiogram of the gel and the quantitative results. Figs. 8 and 9 show the time course of limited tryptic digestion of G_s. When plasma membranes were preincubated with 100 nM Gpp(NH)p and 1 μM glucagon, the sensitivity of the two $G_{s\alpha}$ to trypsin was significantly enhanced. On the other hand, 100 nM Gpp(NH)p and 10 μM isoproterenol enhanced the sensitivity of $G_{s\alpha\text{-}L}$, but not $G_{s\alpha\text{-}S}$, to trypsin. As shown in Figs. 10 and 11, each agonist increased the sensitivity of $G_{s\alpha}$ to trypsin in a concentration-dependent manner.

Next, the effects of the two hormones on the sensitivity of $G_{s\alpha}$ to trypsin were examined in liver plasma membranes prepared from normal male rats (Table IV). As in the partially hepatectomized male rat livers, glucagon facilitated the susceptibility of $G_{s\alpha}$ to trypsin slightly but significantly. In contrast, the effect of isoproterenol on this proteolysis could not be detected. This might have been due to the inefficient coupling of β-adrenergic receptors to G_s in normal male rats (Chapter II). Replacement of isoproterenol by adrenaline gave the same results (data not shown).

DISCUSSION

From the results of the present study, it seems clear that glucagon-induced cAMP generation is increased after partial hepatectomy (Figs. 1 and 2). This increase was not associated with any detectable changes in the number or affinity of glucagon receptors (Table I) or the intrinsic activity of adenylate cyclase (Chapter III). On the other hand, it was accompanied with concomitant increases in the amount of $G_{s\alpha}$ (Chapter III). Taking together, the enhanced responsiveness to glucagon could be primarily accounted for by an increment in the amount of $G_{s\alpha}$. These results (Figs. 1 and 2) were not in accordance with those of the previous reports that glucagon-induced adenylate cyclase activity was not affected by partial hepatectomy (5). However, the present study (Figs. 1-3) has confirmed the concurrent conversion of adrenergic mechanism from an α_1- to a β-type during liver regeneration (4-6). In addition, the present result (Table I) was in contrast to the previous observations that the number of glucagon receptors decreased by partial hepatectomy (7, 8). It should be noted that membrane preparations were stored in liquid nitrogen in the present study. Under these conditions the formation of inside-out vesicles is minimized (24) and the accessibility of hydrophilic radioligands such as [^{125}I]glucagon to the surface receptors would be reduced if inside-out vesicles are formed. The discrepancy between the present and previous results (7, 8) might, therefore, be due to possible differences in the degree of inside-out vesicle formation.

Effects of glucagon and adrenaline on hepatic adenylate cyclase activity were not additive in hepatocytes and liver plasma membranes (Figs. 4 and 5). These results confirmed those of the earlier study which showed that the effects of glucagon and β-adrenergic agonists on glycogenolysis are not additive in fetal hepatocytes (25). It might be

argued that adenylate cyclase activity reaches a maximal level in response to glucagon or a β-adrenergic agonist alone. However, adenylate cyclase activity induced by a combination of forskolin and GTPγS was much higher than that induced by glucagon and/or β-adrenergic agonists (Fig. 5). Forskolin and GTP analogs are known to stimulate adenylate cyclase synergistically (26, 27). The activation of G_s is required for the full expression of forskolin-stimulated adenylate cyclase activity (27). The non-additive actions of the two hormones observed here contrast with the results reported by Bitensky *et al.* (10, 11). It should be noted that they assayed hepatic adenylate cyclase activity in liver homogenates without added GTP (10, 11), because the involvement of G proteins in cAMP production was not known at that time. With low levels of GTP, adenylate cyclase activation stimulated by glucagon or catecholamines is too low for reliable quantification. In this study, assay of the enzyme activity was performed in the presence of nonhydrolyzable GTP analogs. Thus, the discrepancy between the present observations and their reports might be ascribed to the effect of guanine nucleotides on hormone-induced adenylate cyclase activity. These findings suggest that glucagon and β-adrenergic receptors utilize, at least partly, the same signal transduction pathway.

The affinity of receptors for agonists reflects the coupling state of the receptor with G proteins (20, 21). When two different receptor classes compete for the same pool of G proteins, addition of the agonist for one of the receptors will allow that receptor to catalyze the exchange from GDP to GTP on the G proteins and the dissociation of G_α from βγ. Since the dissociated G proteins are no longer associated with the receptors, the other class of receptor is prevented from binding to the G proteins, thereby showing low affinity for its agonists (28). This method was used to examine the possibility that different cAMP-generating receptors share

the same pool of G_s. Glucagon, through its interaction with its receptors, decreased the content of the high-affinity state of β-adrenergic receptors for their agonists without altering the affinity (Table II, Figs. 6 and 7). This inhibitory effect of glucagon might be mediated by protein kinases activated by glucagon receptors. This possibility can be excluded, however, because no ATP, the substrate of protein kinases, was added to liver plasma membrane preparations used for the binding assay. Thus, glucagon receptors appear to share the same G_s with β-adrenergic receptors.

Interestingly, the binding parameters of [^{125}I]glucagon to glucagon receptors were not affected by activation of β-adrenergic receptors (Fig. 8). Two alternative explanations are possible for these results. (A) Glucagon receptors may bind to their G proteins with higher affinity than β-adrenergic receptors. (B) Since the number of glucagon receptors is much higher than that of β-adrenergic receptors, the pool of G_s available for glucagon receptors may be larger than that available for β-adrenergic receptors. Which explanation is more likely remains unknown at present.

The coupling of receptors with G_s was evaluated by the tryptic digestion method (Figs. 9-12). $G_{s\alpha\text{-}L}$ was more susceptible to this protease in the presence of β-adrenergic agonists, whereas the sensitivity of $G_{s\alpha\text{-}S}$ to tryptic cleavage remained unchanged. In contrast, glucagon enhanced not only the trypsinolysis of $G_{s\alpha\text{-}L}$ but also that of $G_{s\alpha\text{-}S}$. Effect of each agonist on the tryptic digestion was dependent on their concentrations. Thus, glucagon receptors appear to catalyze the exchange of guanine nucleotides on both $G_{s\alpha\text{-}S}$ and $G_{s\alpha\text{-}L}$, whereas β-adrenergic receptors do so on $G_{s\alpha\text{-}L}$ preferentially. In conclusion, glucagon receptors share the common $G_{s\alpha\text{-}L}$ with β-adrenergic receptors, but are also coupled to the another $G_{s\alpha}$, $G_{s\alpha\text{-}S}$, in partially hepatectomized male rat livers.

Table I

Kinetic parameters for specific [^{125}I]glucagon and [^{125}I]ICYP binding to liver plasma membranes

	Glucagon receptor		β-adrenergic receptor	
	Bmax (fmol/mg protein)	K_d value (pM)	Bmax (fmol/mg protein)	K_d value (pM)
No treatment	2224 ± 285	1028 ± 4	72 ± 5	129 ± 25
Partial hepatectomy	2000 ± 123	902 ± 103	245 ± 21*	91 ± 15

Liver plasma membranes were prepared on 0 or 2nd day after partial hepatectomy. [^{125}I]Glucagon or [^{125}I]ICYP binding in liver plasma membranes were performed. Bmax and K_d values were obtained from Scatchard plots. Values are the mean ± S.E. from 4 separate experiments. **P<0.01 for values of partially hepatectomized rats *versus* normal from t test.

Table II

Kinetic parameters for specific isoproterenol binding to liver plasma membranes

	None	GTPγS	GTPγS + Glucagon
K_L (nM)	450 ± 23	445 ± 18	452 ± 21
K_H (nM)	6.3 ± 1.5	6.8 ± 1.2	6.1 ± 1.3
K_L/K_H	73 ± 4	65 ± 3	74 ± 4
R_L (%)	24 ± 4	49 ± 4	73 ± 4*
R_H (%)	76 ± 5	51 ± 5	27 ± 3*

Membranes were prepared on 2nd day after partial hepatectomy. Competition experiments were carried out as outlined in Fig. 6. The parameters were calculated by Scatchard analysis. Values are the mean ± S.E. of 4 separate experiments. *P<0.01 for values in the presece of glucagon and 10 nM GTPγS *versus* in the presence of 10 nM GTPγS alone from t test.

Table III

Effects of guanine nucleotides and hormones on proteolysis of $G_{s\alpha}$ by TPCK-trypsin

	No treatment		Partial hepatectomy	
	42 kDa (%)	47 kDa (%)	42 kDa (%)	47 kDa (%)
None	105 ± 6	90 ± 5	102 ± 4	83 ± 4
Gpp(NH)p	91 ± 4	79 ± 6	87 ± 3	72 ± 3
Gpp(NH)p +isoproterenol	86 ± 6	70 ± 4	89 ± 4	52 ± 2**
Gpp(NH)p +glucagon	75 ± 5*	54 ± 3**	66 ± 4**	48 ± 2**
Gpp(NH)p*	71 ± 6**	49 ± 5**	59 ± 4**	38 ± 3**

Liver plasma membranes were prepared on 2nd day after partial hepatectomy. Membranes were preincubated with 100 nM Gpp(NH)p, 10 μM isoproterenol, 1 μM glucagon for 20 min, 100 μM Gpp(NH)p* for 60 min or without addition (None) for 20 min at 30 °C. After trypsin treatment for 1 min at 30 °C , the samples were subjected to SDSPAGE. Control values (100 %) were detected in the absence of trypsin. Values are mean ± S.E. for 9 separate experiments.
** P < 0.01, * P < 0.05 *versus* to values in the presence of 100 nM Gpp(NH)p alone from t test.

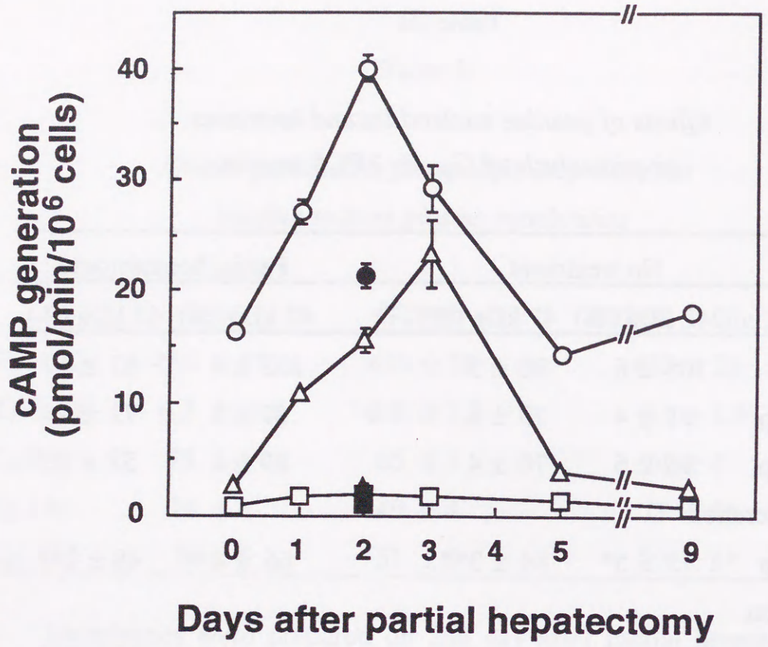

Fig. 1. **Glucagon- or adrenaline-induced cAMP accumulation in hepatocytes after partial hepatectomy.** Hepatocytes were isolated from partially hepatectomized (○, △, □) or sham-operated rats (●, ▲, ■). Hepatocytes were incubated with 1 μM glucagon (○, ●), 10 μM adrenaline (△, ▲) or without addition (□, ■). Values are the mean ± S.E. for 4 separate experiments.

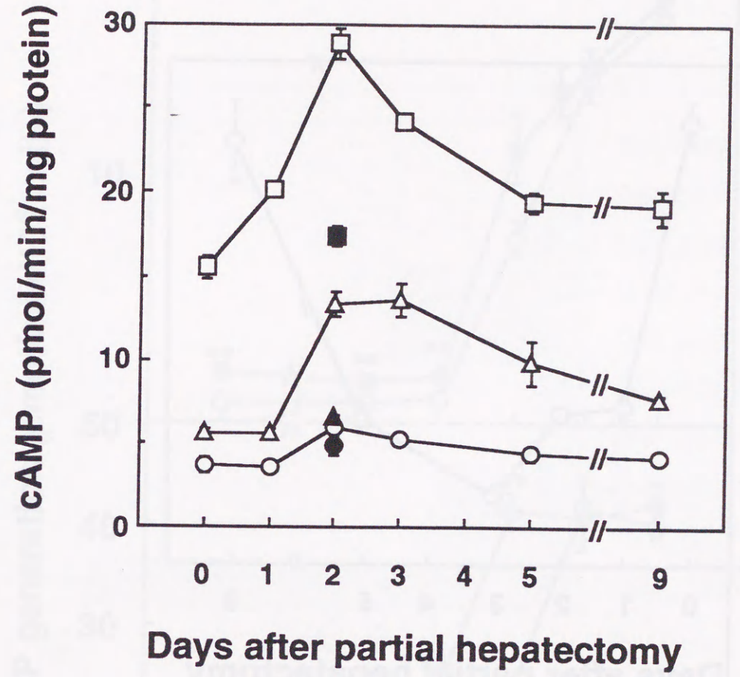

Fig. 2. **Glucagon- or adrenaline-induced cAMP generation in liver plasma membranes after partial hepatectomy.** Liver plasma membranes were prepared from partially hepatectomized (○, △, □) or sham-operated rats (●, ▲, ■). Membranes were incubated in the presence of 100 nM GTPγS with 1 μM glucagon (□, ■), 10 μM adrenaline (△, ▲) or without addition (○, ●). Values are the mean ± S.E. for 4 separate experiments.

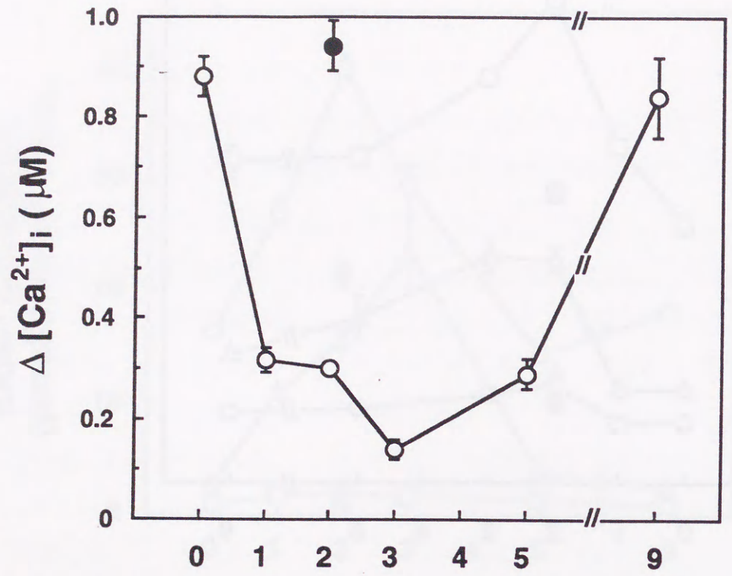

Fig. 3. **Adrenaline-induced elevation of $[Ca^{2+}]_i$ in hepatocytes after partial hepatectomy.** Hepatocytes from partially hepatectomized (○) or sham-operated rats (●) were preincubated with Fura-2 AM (5 μM) for 15 min at 37 °C. $[Ca^{2+}]_i$ was measured when 10 μM adrenaline was added to the incubation medium. Values are the mean ± S.E. for 4 separate experiments.

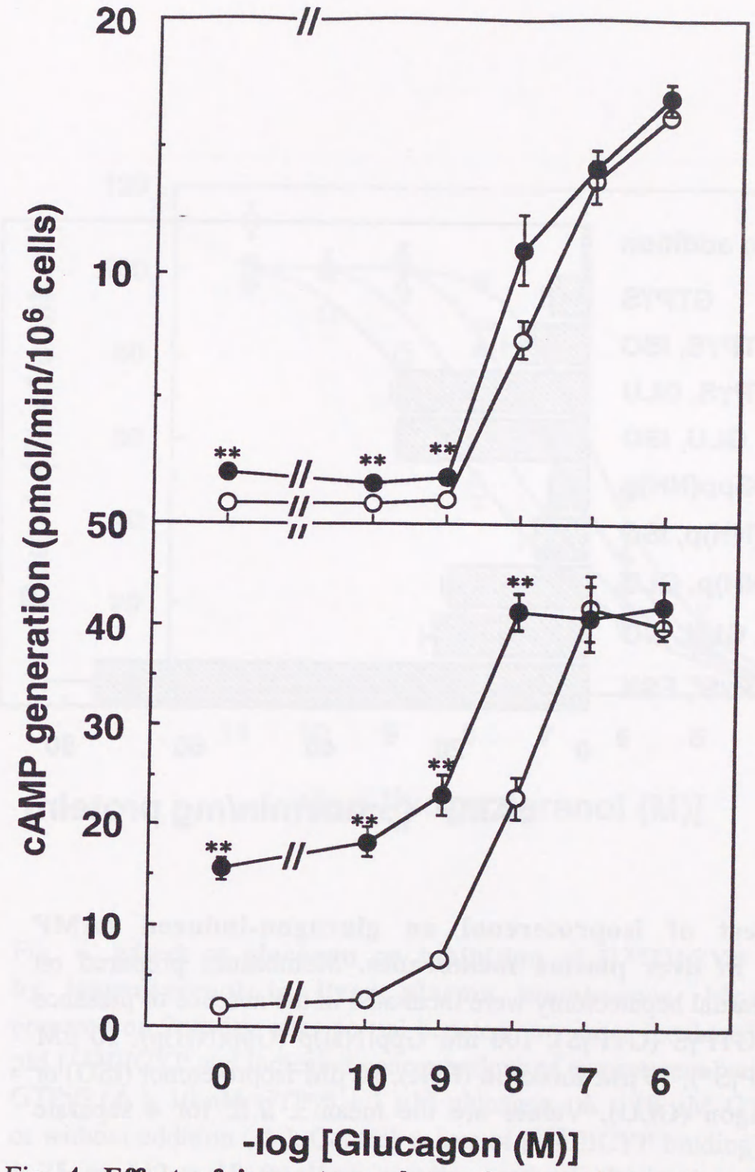

Fig. 4. **Effect of isoproterenol on glucagon-induced cyclic AMP accumulation in rat hepatocytes.** Hepatocytes from rat livers on 0 (top panel) or 2nd day (bottom panel) after partial hepatectomy were incubated in the presence (●) or absence (○) of 10 μM isoproterenol with indicated concentrations of glucagon. Values are the mean ± S.E. for 4 separate experiments. **P<0.01, values in the presence of isoprpterenol *versus* in the absence of isoproterenol from t test.

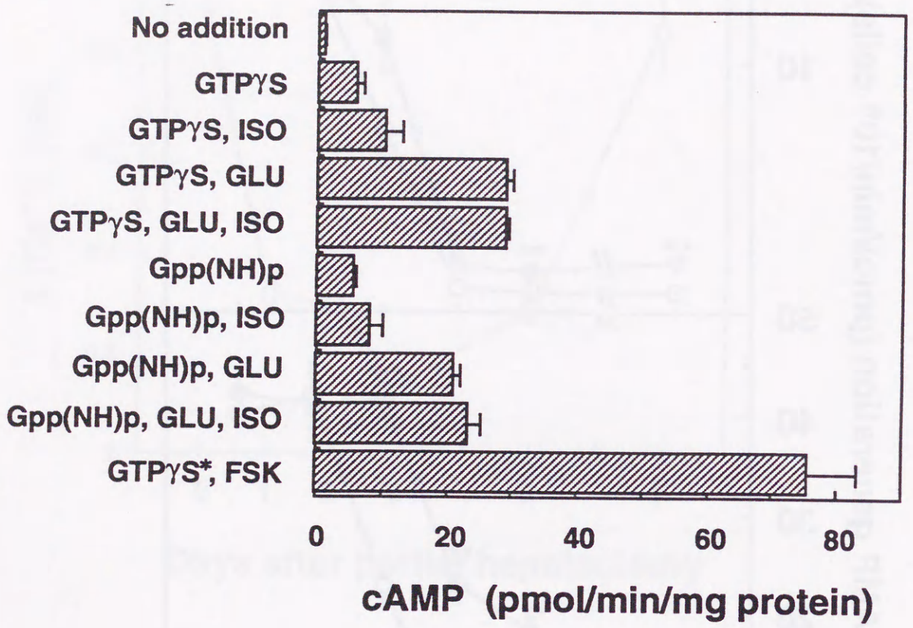

Fig. 5. **Effect of isoproterenol on glucagon-induced cAMP generation in liver plasma membranes.** Membranes prepared on day 2 after partial hepatectomy were incubated in the absence or presence of 100 nM GTPγS (GTPγS), 100 nM Gpp(NH)p (Gpp(NH)p), 10 μM GTPγS (GTPγS*), 10 μM forskolin (FSK), 10 μM isoproterenol (ISO) or 1 μM glucagon (GLU). Values are the mean ± S.E. for 4 separate experiments.

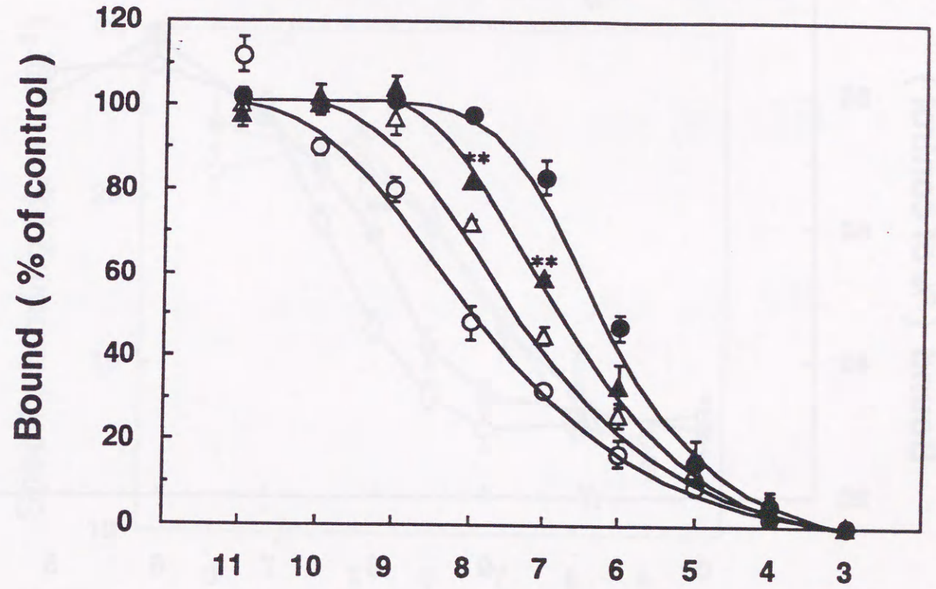

Fig. 6. **Effect of glucagon on inhibition of [^{125}I]ICYP binding by isoproterenol in liver plasma membranes.** Membranes prepared on 2nd day after partial hepatectomy were incubated with 100 pM [^{125}I]ICYP and indicated concentrations of isoproterenol with 10 nM GTPγS (Δ), 10 nM GTPγS + 1 μM glucagon (▲), 10 μM GTPγS (●) or without addition (○). Control values of [^{125}I]ICYP binding were 123 ± 25 or 142 ± 18 fmol/mg protein, respectively in the absence or presence of GTPγS. Values are the mean ± S.E. for 4 separate experiments. **P<0.01 for values in the presence of glucagon and 10 nM GTPγS *versus* in the presence of 10 nM GTPγS from t test.

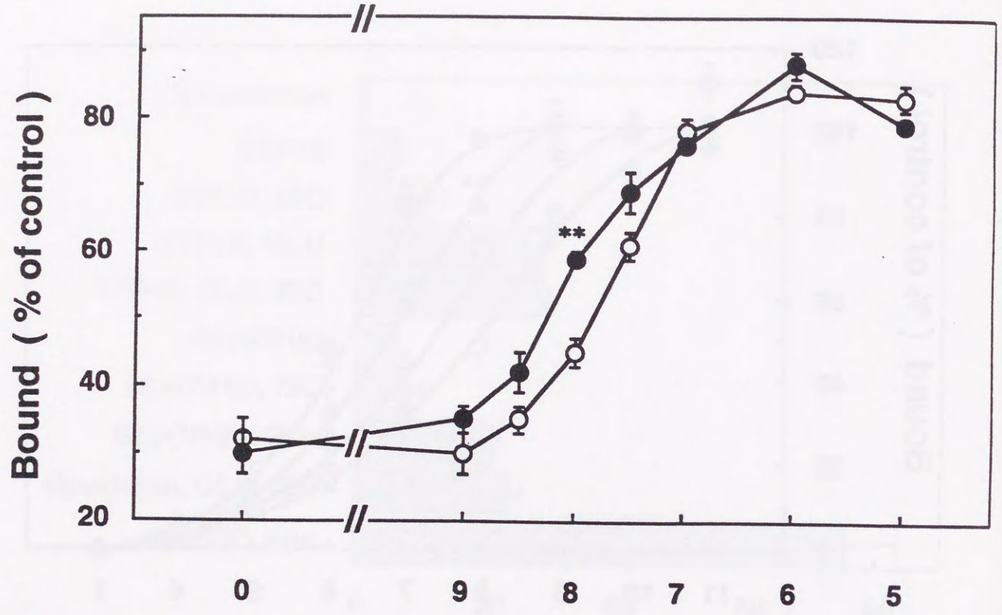

Fig. 7. **Effect of GTPγS on inhibition of [^{125}I]ICYP binding by isoproterenol in liver plasma membranes.** Membranes prepared on 2nd day after partial hepatectomy were incubated in the presence (●) or absence (○) of 1 μM glucagon with 100 pM [^{125}I]ICYP, 10 nM isoproterenol and indicated concentrations of GTPγS. Other conditions were the same as described under the legend of Fig. 6. Values are the mean ± S.E. for 4 separate experiments. **P<0.01 for values in the presence of glucagon *versus* in the absence of glucagon from t test.

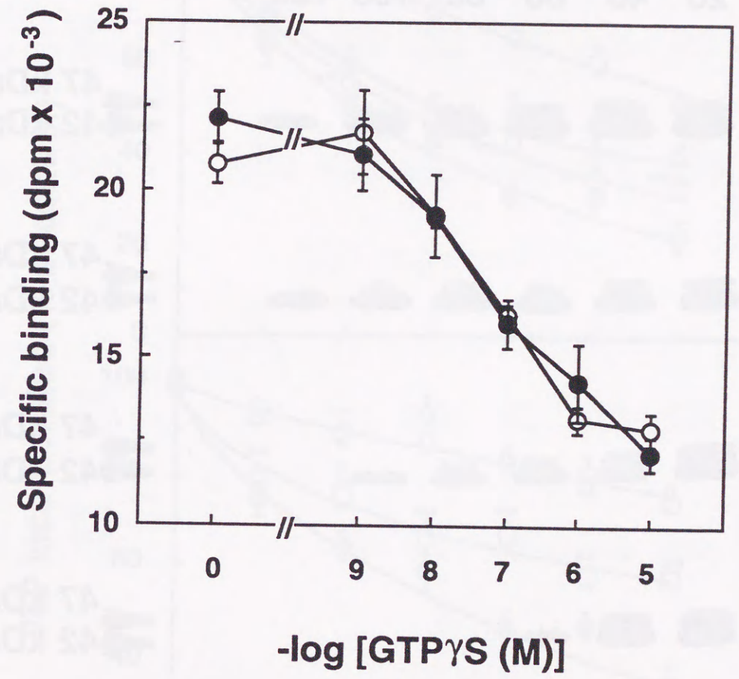

Fig. 8. **Effect of isoproterenol on glucagon binding to liver plasma membranes.** Liver plasma membranes were prepared on 2nd day after partial hepatectomy. Membranes were incubated in the presence (●) or absence (○) of 10 μM isoproterenol with 300 pM [^{125}I] glucagon and indicated concentrations of GTPγS. Values are the mean ± S.E. for 4 separate experiments.

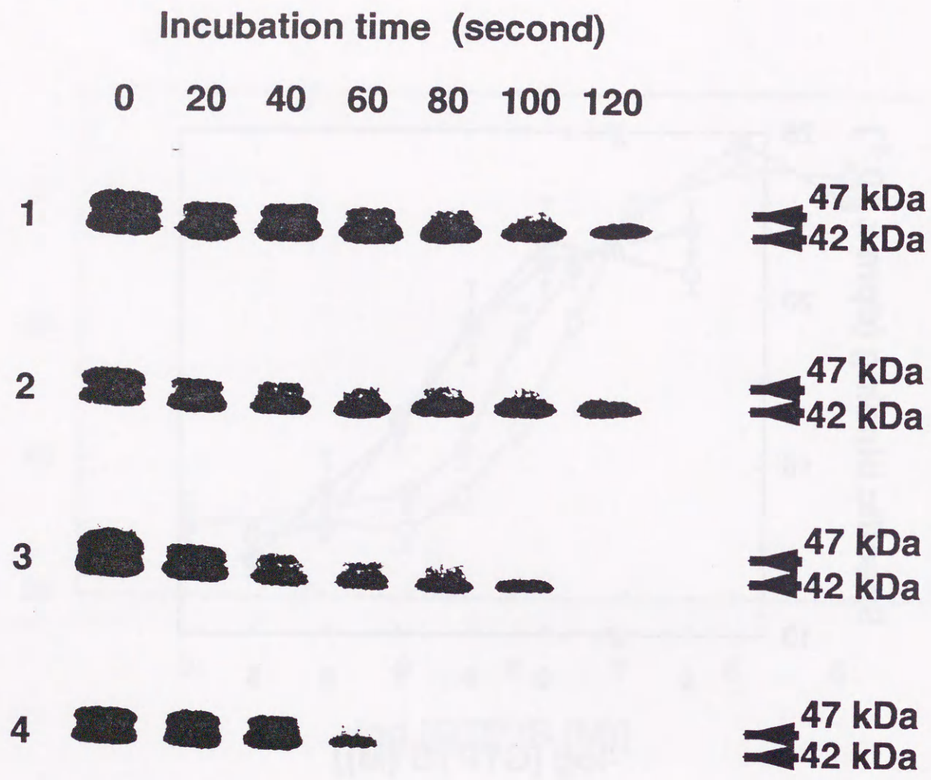

Fig. 9. **Autoradiogram of [^{32}P]ADP-ribosylated $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$ after TPCK-trypsin treatment.** Membranes prepared on 2nd day after partial hepatectomy were preincubated with 100 nM Gpp(NH)p (lane 1), 100 nM Gpp(NH)p + 10 μM isoproterenol (lane 2), 100 nM Gpp(NH)p + 1 μM glucagon (lane 3) for 20 min or with 100 μM Gpp(NH)p for 60 min (lane 4). Membranes were incubated with trypsin, treated with cholera toxin to introduce ADP-ribosyl moiety, and subjected to SDS-PAGE.

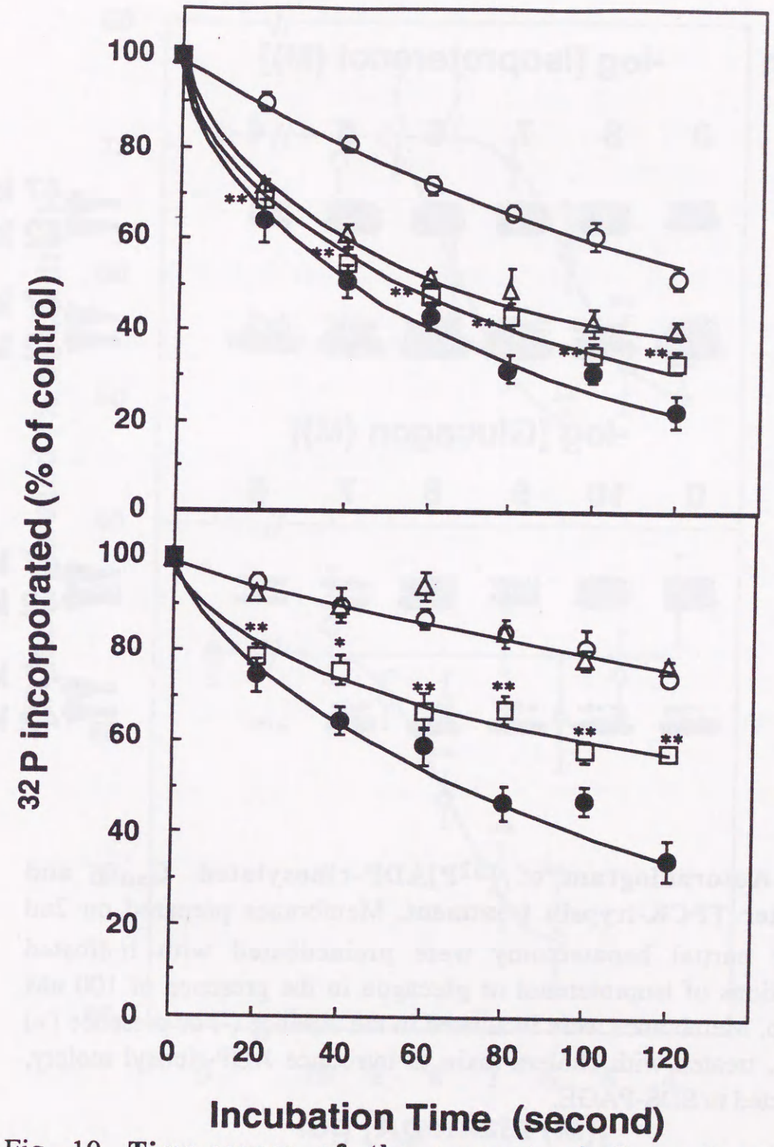

Fig. 10. **Time-course of the amount of $G_{s\alpha}$ after tryptic digestion.** [^{32}P]ADP-ribosylated $G_{s\alpha\text{-}L}$ (top panel) and $G_{s\alpha\text{-}S}$ (bottom panel) after TPCK-trypsin treatment in Fig. 9 were quantified. Membranes were preincubated with 100 nM Gpp(NH)p (○), 100 nM Gpp(NH)p + 10 μM isoproterenol (△), 100 nM Gpp(NH)p + 1 μM glucagon (□) for 20 min or with 100 μM Gpp(NH)p for 60 min (○). Values are the mean ± S.E. for 9 separate experiments. **P<0.01, *P<0.05 for values in the presence of hormone and 100 nM GTPγS *versus* in the presence of 100 nM GTPγS from t test.

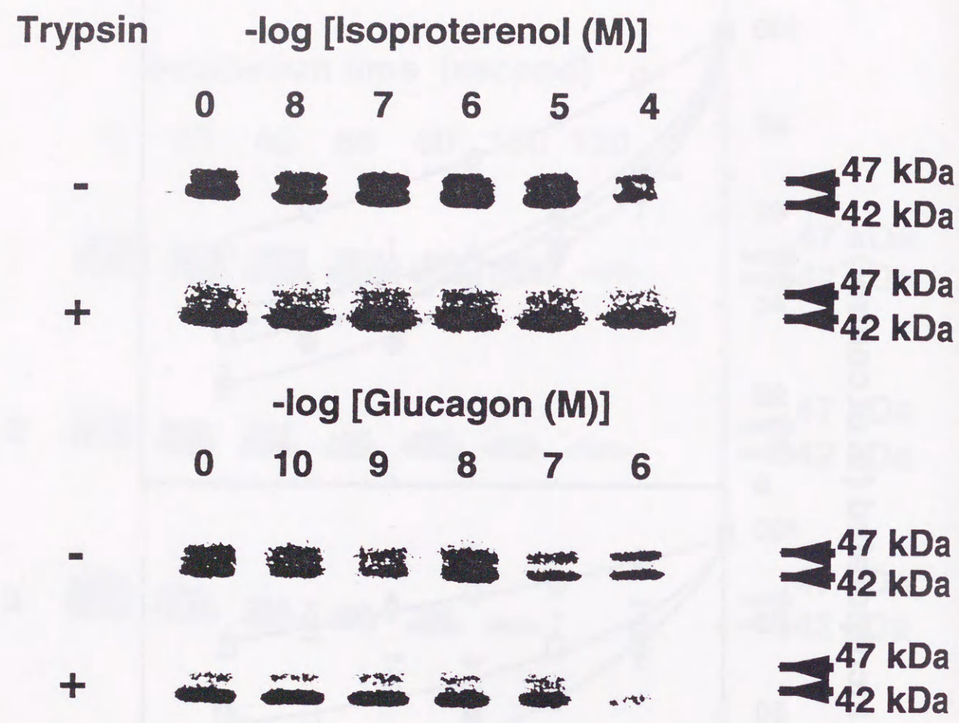

Fig. 11. **Autoradiogram of [^{32}P]ADP-ribosylated $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$ after TPCK-trypsin treatment.** Membranes prepared on 2nd day after partial hepatectomy were preincubated with indicated concentrations of isoproterenol or glucagon in the presence of 100 nM Gpp(NH)p. Membranes were incubated in the absence (-) or presence (+) of trypsin, treated with cholera toxin to introduce ADP-ribosyl moiety, and subjected to SDS-PAGE.

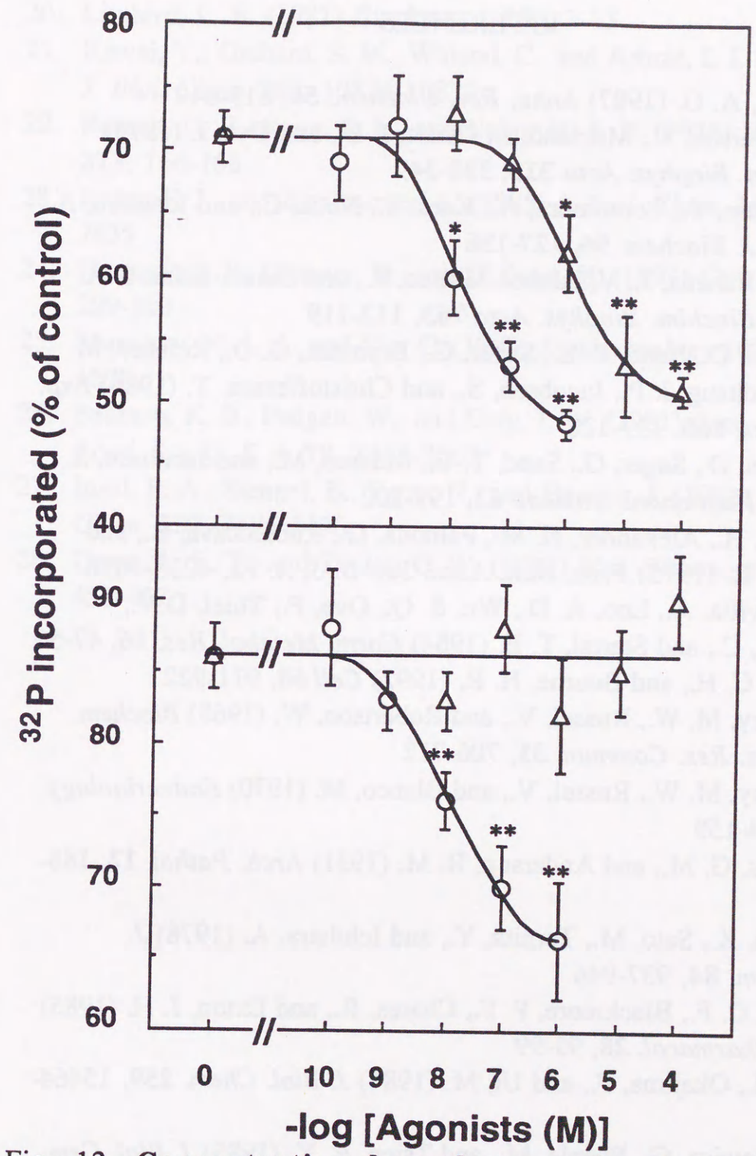

Fig. 12. **Concentration-dependency of glucagon and isoproterenol on tryptic digestion of $G_{s\alpha}$.** $G_{s\alpha\text{-}L}$ (top panel) and $G_{s\alpha\text{-}S}$ (bottom panel) after TPCK-trypsin treatment in Fig. 11 were quantified. Membranes were preincubated with isoproterenol (Δ) or glucagon (○) in the presence of 100 nM Gpp(NH)p. Control values were detected in the absence of trypsin. Values are the mean ± S.E. for nine separate experiments. *P < 0.05 or **P < 0.01 for values in the presence of hormones *versus* in the absence of hormones from by t test.

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Chapter V

Comprehensive Discussion

In the present study, the sexual dimorphism of β-adrenergic agonists sensitive adenylate cyclase activity can be accounted for the sex-difference in β-adrenergic receptors, G_s and their interaction in rat liver (Chapter II). Mammalian liver is responsible to androgen and estrogen (1). Liver contains receptors for these hormones (2, 3) and responds by the synthesis of specific protein (1), resulting in the sexual dimorphic hepatic content of certain receptors and microsomal enzymes (4-6). Besides β-adrenergic receptors (4), there are sex difference in epidermal growth factor receptors (5) and cytochrome P-450 (CYP) (6). These sexual dimorphisms are ascribed to the endocrine state of various hormones (4). The exact physiological significance of these sexual dimorphism remains unclear, however. If speculation was permitted, the significance of sex difference in β-adrenergic agonists-sensitive adenylate cyclase activity would be discussed.

The liver plays a central role in homeostasis of blood glucose through gluconeogenesis and glycogenolysis. Adrenaline stimulates these process through α_1- and β-adrenergic receptors (7). Adrenaline-stimulated adenylate cyclase activity of rat liver decreases markedly during early development (8, 9). The responsiveness of liver adenylate cyclase to β-adrenergic agonists is maximal at 5-10 days of life and then decreases progressively during the remainder of the developmental life span (8, 9). In young adult male rats, the adrenaline-stimulated enzyme activity is almost negligible (9). On the other hand, in the female, β-adrenergic effect of adrenaline is not lost entirely (10). Thus, there is a sexual dimorphism of hepatic β-adrenergic responsiveness in mature rats (10). The emergence of β-adrenergic response in the fetus appears to be associated proliferation of hepatocyte, and that in the female dose to be associated with gestation. Since cell growth during development requires

energy, it gives rise to a possibility that β-adrenergic receptor plays an pivotal role in the energy supplying process of rat liver.

Besides glucose metabolism, the liver plays a central role in the metabolism of endogenous and exogenous compounds including many steroids, drugs, and chemical carcinogens. These compounds are metabolized in the hepatocyte to water-soluble end products which are then secreted into the bile and urine (11, 12). While glucose metabolism in the liver is an energy supplying process, steroid metabolism is an energy consuming one. In hepatocytes, however, the two metabolisms are influenced by the activity of the mixed function oxidase system and need NADPH for oxidation-reduction in common (13). If one pathway (*e.g.* glucose metabolism) is altered, there might be changes in the metabolism of the other way (steroid metabolism). Actually, steroid metabolism is reduced in non-insulin dependent diabetes mellitus (14). CYP is involved in steroid metabolism and comprises a super family of hem protein monooxygenase enzymes. Expression of CYP in the liver and other tissues is modulated by exposure to foreign compounds, many of which can differentially induce and suppress the levels of individual CYP enzymes (15). Moreover, hormonal regulation of CYP enzyme activity is also suggested by studies on the modulation of CYP-dependent monooxygenase activity in the liver by the cAMP analog dibutiryle cAMP (16, 17) as well as by hormones known to elevate cAMP levels within cells (18). Indeed, the addition of adrenaline to primary rat hepatocytes causes an increased phosphorylation of CYP2E1 on Ser^{129}, which is located within the cAMP-dependent protein kinase recognition sequence (19). CYP2E1 is ethanol-induced type of CYP. Moreover, adrenaline also increases the rate of CYP2E1 degradation at similar concentrations as needed for phosphorylation of the protein. Thus, it seems likely that β-adrenergic receptor may reduce steroid metabolism

by the enzyme degradation following phosphorylation. Taken together, it is proposed that β-adrenergic receptors may increase the supply of energy during development by the stimulation of glucose metabolism and the attenuation of steroid metabolism in rat liver. Further studies are required to prove this hypotheses *in vivo*.

The present study suggested that estrogen, rather than androgen, might be involved in the sex- and partial hepatectomy-dependent appearance of β-adrenergic responsiveness (Chapters II and III). A fairly close correlation between estrogen and β-adrenergic adenylate cyclase system is also observed in other tissues including mammary gland (20) and granulosa cell (21). The next problem is the plausible mechanism by which the emergence of β-adrenergic agonist-sensitive hepatic adenylate cyclase activity is induced by estrogen. In this regard, there are several physiological effects of estrogen. (A) Estrogen can regulate the level of β-adrenergic receptors. In rat mammary gland, the number of β-adrenergic receptors changes in parallel with alterations in β-adrenergic responsiveness during estrus cycle (20). (B) Estrogen is able to induce the expression of $G_{s\alpha}$. In castrated female rats, estrogen treatment induces a significant increase in pituitary $G_{s\alpha}$ (22). (C) The hormone is capable of facilitating the coupling of β-adrenergic receptors with G_s. In weanling or castrated adult male rat livers, the treatment with estrogen elevates the β-adrenergic response accompanied with the enhanced coupling of β-adrenergic receptor with G_s (23). Whether genes of β-adrenergic receptors and $G_{s\alpha}$ have the estrogen responsible element is yet unclear, however. Moreover, estrogen can affect β-adrenergic responsiveness indirectly through alterations in the lipid composition of plasma membranes and serum level of thyroid hormone. (D) Estrogen produces significant increases in the cholesterol ester content and cholesterol-to-phospholipid molar ratio of rat liver plasma membranes (24, 25). In

reconstituting experiments, phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and α-tocopherol are all required to β-adrenergic receptor-mediated activation of G_s by GTPγS (26). Cholesterol further enhances the receptor-mediated activity of this system (27). (E) Estrogen-administration of adult male rats decreases the serum level of thyroid hormone (28). Hypothyroidism potentiates β-adrenergic stimulation of adenylate cyclase activity in rat livers (29). In thyroidectomized rats, the number of β-adrenergic receptors (30) and β subunit of G protein (31) are increased. Thus, it seems likely that estrogen may modulate β-adrenergic adenylate cyclase system through the induction of the protein composed of the system and/or environmental changes around the system.

The present study indicated that $G_{s\alpha\text{-}L}$ was preferentially coupled to β-adrenergic receptors and $G_{s\alpha\text{-}S}$ could be coupled to both β-adrenergic and glucagon receptors (Chapters III and IV). $G_{s\alpha\text{-}L}$ and $G_{s\alpha\text{-}S}$ are identical except that $G_{s\alpha\text{-}L}$ contains an inserted region of 15 residues (32). This region (from Gly^{72} to Gly^{86}) is unique to $G_{s\alpha}$. What kind of role does the unique region play in the function of G protein? cDNA sequences predict the primary structures of α subunit of seven G proteins including G_s, G_o three proteins currently classified as G_i, and the two distinct transducins of retinal rod and cone cells. Comparison of these amino acid sequences, aligned with a hypothetical "average " G_α (33), reveals a constituent pattern of conserved and divergent sequences (Fig. 1, *top*). According to Halliday's nomenclature (34), the homologous stretches of sequence are designated A, C, E and G (Fig. 1, *bottom*). These conserved regions probably perform similar or identical function in the different G_α. Halliday proposes that these conserved sequences would play important roles in the structures of the guanine nucleotide-binding site. Recently, X-ray analysis implicates that domain B, C and G

locate near the γ phosphate of Mg^{2+}-GTP binding to $G_{t\alpha}$ (35). Conversely, it is tempting to imagine that divergent sequences reflect specific and distinct functions of individual G proteins, such as specific interactions with different sets of receptors and effectors. Domain I (the NH_2-terminal of G_α) is hydrophilic and contains the site for limited tryptic digestion (36, 37). Although this region may be involved in the interaction with βγ subunits (36, 37), its precise function has not yet been shown. With mutagenesis approach, it is indicated that the NH_2-terminal of domain III is required for activation of adenylate cyclase (38, 39). The COOH-terminal of Domain III has been suggested to be the region involved in coupling of the G protein with receptors by following studies. First, the uncoupling of G_i and G_o type G protein from receptors after ADP-ribosylation by pertussis toxin, which modifies a cystein residue, the fourth amino acid from the COOH-terminal (40). Second, the *unc* mutant of G_s, which cannot couple with receptors, has also been shown to be altered in the COOH-terminal region of the protein (41). Finally, antibodies raised against COOH-terminal peptides have been shown to inhibit the receptor activation of G proteins (42). Domain II is the most structural divergent domain, but the functional significance of this domain is not yet clear. In this regard, the present study exhibited a possible role of the domain II in the function of G_α. The inserted region by alternative splicing of the $G_{s\alpha}$ gene is located in this domain. $G_{s\alpha\text{-}L}$ appears to be coupled with receptors more specifically than $G_{s\alpha\text{-}S}$ (Chapters III and IV). Thus, even if domain II is not a major point of receptor-G protein interaction, this domain may be involved in modulation of their interaction and specificity of individual G_α for receptors.

In partially hepatectomized male rat livers, glucagon receptors share the common $G_{s\alpha\text{-}L}$ with β-adrenergic receptors, but are also coupled to

the another G_s, $G_{s\alpha\text{-}S}$ (Chapters III and IV). This accounts for the high potency of glucagon in stimulating adenylate cyclase, compared with catecholamines. In response to the emergence of β-adrenergic receptor-mediated function, the ratio of $G_{s\alpha\text{-}L}$ to $G_{s\alpha\text{-}S}$ was greater in the female than in the male (Chapter II). Moreover, in normal male rat hepatocytes, cAMP accumulation in response to glucagon and adrenaline did not occur additively (Chapter IV). Furthermore, in spite of the failure to detect coupling of β-adrenergic receptors with G_s, the tryptic digestion method suggested that glucagon receptors are coupled with the two forms of G_s in normal male rat livers (Chapter IV). Thus, the coupling of β-adrenergic and glucagon receptors with G_s in normal rat livers appears to be similar to that in partially hepatectomized rat livers.

In the present study, a new method of tryptic digestion was used to evaluate coupling of receptors with G_s (Chapters III and IV). The most common approach to determine which G protein(s) are coupled with a given receptor is to use purified or recombinant G proteins to reconstitute receptor function (43, 44). However, it is not easy to determine a specific function of a G protein of defined molecular composition because of difficulties in removing closely related proteins, contamination of unidentified proteins and the absence of cellular factors that play a role in the determination of specificity. Since a trypsinization approach dose not require the cloning, expression, and purification of receptors and G protein, this approach is useful to analyze receptor-G protein interaction qualitatively in liver membranes. Because of its simplicity and reliability, this method has a great potentiality.

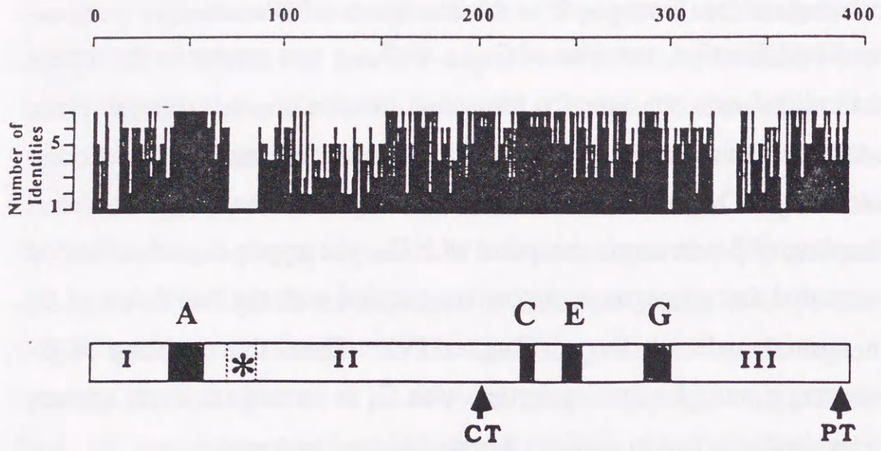

Fig. 1 **Conservation of primary structure and domain of G_α (Ref. 35).** The graph at the top plots amino acid residue number in a composite G_α, "average" $G\alpha$ *versus* the number of identical residues at each position among the seven G_α whose amino acid sequences are known (the two isoforms of $G_{s\alpha}$, the three of $G_{i\alpha}$ and the two of $G_{t\alpha}$). The bar at the bottom shows, on the same axis, the sites ADP-ribosylated by cholera (CT) and pertussis (PT) toxins, the location of the peptides sequences inserted by alternative splicing of the $G_{s\alpha}$ gene (*), the locations of four regions that contribute to the GDP-binding domain (designated A, C, E and G), and three additional presumed domains (I, II, III) of the G_α.

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SUMMARY

Alterations in stimulatory G protein associated with the sex-dependent appearance of β-adrenergic receptor-mediated function in rat liver

In rat hepatocytes, β-adrenergic receptor-mediated cAMP generation was higher in the female than in the male. The level of β-adrenergic receptors, detected by [^{125}I]iodocyanopindolol, was elevated in the female. In agonist competitive experiments, coupling of β-adrenergic receptors with G_s was enhanced in the female. The amount of $G_{s\alpha}$, quantified using ADP-ribosylation catalyzed by cholera toxin, was increased in the female. The ratio of $G_{s\alpha\text{-}L}$ to $G_{s\alpha\text{-}S}$ was increased in the female. There was no significant difference in the intrinsic activity of adenylate cyclase in either sex. In addition, estrogen potentiated β-adrenergic function in both sexes, whereas castration or testosterone had no effect on this function. These results indicate that alterations in β-adrenergic receptors, G_s and the interaction between receptors and Gs are closely associated with the sex-dependent appearance of β-adrenergic function. Moreover, estrogen appears to play an important role on this appearance of β-adrenergic function.

Alterations in stimulatory G protein associated with the partial hepatectomy-dependent appearance of β-adrenergic receptor-mediated function in rat liver

In male rat hepatocytes and liver plasma membranes, β-adrenergic receptor-mediated cAMP generation was dramatically stimulated after partial hepatectomy. The number of β-adrenergic receptors, detected by [^{125}I]iodocyanopindolol, was increased following partial hepatectomy. In agonist competition experiments, the coupling of β-adrenergic receptors

with G_s was enhanced after surgery. The amount of $G_{s\alpha}$, quantified using ADP-ribosylation catalyzed by cholera toxin, was increased following partial hepatectomy. In addition, there was a significant increase in the level of $G_{s\alpha}$ mRNA prior to that of $G_{s\alpha}$ protein. Partially hepatectomized rats showed a significant increase in the ratio of $G_{s\alpha\text{-}L}$ to $G_{s\alpha\text{-}S}$. There was no significant alteration in the intrinsic activity of adenylate cyclase throughout regeneration. In addition, anti-estrogen agent inhibited partial hepatectomy-dependent appearance of β-adrenergic responsiveness. With a method using tryptic digestion, coupling of β-adrenergic receptors with G_s was evaluated. The GTP-bound forms of both $G_{s\alpha\text{-}S}$ and $G_{s\alpha\text{-}L}$ were more trypsin-sensitive than their GDP-bound forms. In the presence of GTP analog, β-adrenergic agonists increased the sensitivity of $G_{s\alpha\text{-}L}$ to trypsin, but not $G_{s\alpha\text{-}S}$. This effect of agonists was retarded by the addition of the β-adrenergic antagonist. These results indicate that alterations in β-adrenergic receptors, G_s and their interaction are closely associated with the partial hepatectomy-dependent appearance of β-adrenergic function. Furthermore, β-adrenergic receptors are preferentially coupled with $G_{s\alpha\text{-}L}$. Moreover, it seems likely that estrogen plays an important role on this appearance of β-adrenergic function.

Coupling of glucagon receptors with stimulatory G protein in partially hepatectomized rat livers

In male rat hepatocytes and liver plasma membranes, glucagon receptor-mediated cAMP generation was increased after partial hepatectomy. The number of glucagon receptors, detected by [^{125}I]glucagon, was not significantly altered after operation. In partially hepatectomized male rat livers, effects of glucagon and β-adrenergic agonists on hepatic adenylate cyclase activity were not additive. In the

presence of GTP analog, glucagon decreased the apparent affinity of β-adrenergic receptors for their agonists. On the other hand, in the presence of GTP analog, β-adrenergic agonists did not affect the affinity of glucagon receptors for glucagon. With a method using tryptic digestion, coupling of glucagon receptors with G_s was evaluated. In the presence of GTP analog, β-adrenergic agonists increased the sensitivity of $G_{s\alpha\text{-}L}$ to trypsin in a concentration-dependent manner. On the other hand, in the presence of GTP analog, glucagon increased the sensitivity of both $G_{s\alpha\text{-}S}$ and $G_{s\alpha\text{-}L}$ to trypsin in a concentration-dependent manner. It is proposed that glucagon receptors share the common $G_{s\alpha\text{-}L}$ with β-adrenergic receptors, but are also coupled to the another $G_{s\alpha}$, $G_{s\alpha\text{-}S}$, in partially hepatectomized male rat livers.

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List of Publications

(1) Papers Related to the Present Study

1. Sex Difference in Adrenergic Receptor-Mediated Glycogenolysis in Rat Livers.
   Yagami, T., Tohkin, M., and Matsubara, T.
   Jpn. J. Pharmacol. **54** (1990) 365-374

2. Alterations in the Stimulatory G Protein of the Rat Liver after Partial Hepatectomy.
   Yagami, T., Kirita, S., Matsushita, A., Kawasaki, K., and Mizushima, Y.
   Biochim. Biophys. Acta, **1222** (1994) 81-87

3. Increase in Stimulatory G Proteins and Glucagon-Responsive Adenylate Cyclase Activity in Rat Liver Following Partial Hepatectomy.
   Yagami, T.
   Biochem. Mol. Biol. Int. **32** (1994) 159-166

4. The Involvement of the Stimulatory G Protein in Sexual Dimorphism of β-Adrenergic Receptor-Mediated Functions in Rat Liver.
   Yagami, T., Tohkin, M. and Matsubara, T.
   Biochim. Biophys. Acta, **1222** (1994) 257-264

5. Coupling of Glucagon Receptors to the Small and Large Forms of Stimulatory G Proteins in Partially Hepatectomized Rat Livers.
   Yagami, T.
   in preparation

(2) Other Published Papers:

1. Mastoparan, a Peptide Toxin from Wasp Venom, Stimulates Glycogenolysis Mediated by an Increase of the Cyctosolic Free Ca^{2+} Concentration but not by an Increase of cAMP in Rat Hepatocytes

Tohkin, M., Yagami, T. and Matsubara, T.
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2. Possible Interaction of α_1-Adrenergic Receptor with Pertussis Toxin-Sensitive Guanine-Nucleotide-Binding Regulatory Proteins (G proteins) Responsible for Phospholipase C Activation in Rat Liver Plasma Membranes
Tohkin, M., Yagami, T., Katada, T. and Matsubara, T.
Eur. J. Biochem. **194** (1990) 81-87